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Velia Siciliano
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Cancer Immunotherapy

Methods and Protocols

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Cancer Immunotherapy

Methods and Protocols

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Preface: Cancer Immunotherapy

Over the past years, immunotherapy has emerged as a ground-breaking technology for cancer treatment, in the attempt of removing the breaks that hold the full potential of immune cells activity for targeted tumor cell killing.

A variety of approaches have been developed to date, from the design of organoid models that mimic disease state to adoption of cell engineering approaches where immune cells are equipped with novel or more robust capabilities for targeted immune response. By bringing together some of the most prominent scientists in the field, this book aims at providing an overview of the different areas that are converging to develop the next generation of immunotherapy treatments. The book will walk the readers across state-of-the-art methods for the analysis and characterization of the interactions between tumor and immune cells, and cell engineering tools for cancer treatment, to provide a unique and compelling set of techniques instrumental to work with, and engineer, immune cells.

Challenges are still standing in the effort for a clear understanding of the interactions occurring among the different players within the tumor microenvironment, critical to develop improved therapies.

Chapters 1 and 2 introduce flow cytometry-based assays to isolate immune cells from the tumor microenvironment or to evaluate cancer cell immunogenicity of ovalbumin (OVA)-specific CD8 OT-1 T cells exposed to OVA-expressing MCA205 sarcoma cells.

In Chap. 3, a T-cell receptor specific for melanoma antigen gp100 is presented and its killing capabilities characterized by the xCELLingence system for real time cell analysis.

Chapter 4 describes a protocol for the generation and expansion of CAR T cells using primary mouse T cells.

Novel methodologies that support better understanding and characterization of the features of the tumor environment and the interaction of the different cellular types within it are awaited. Chapters 5, 6 and 7 focus on this, with Chap. 5 introducing in situ decellularization of tissues as a mean to enable the mapping of the extra cellular matrix that is key in supporting tumor growth in vivo, while in Chap. 6, piezoelectric stimulation is described as a novel approach to study the migration and invasion ability of tumor cells. Chapter 7 describes protocols to characterize the physical-chemical properties and therapeutic potential of nanoparticles in vitro on three-dimensional (3D) tumor spheroids.

Microfluidics has witnessed tremendous development over the last decade and application across different fields. In immunotherapy, microfluidics can be adopted for the live analysis of cell-to-cell interactions but also for miniaturization of experiments decreasing the need for starting material, often difficult to isolate. This is the focus of Chap. 8 where a novel microfluidic platform is presented that exposes cancer cells to a linear increasing concentration of a given cytotoxic agent, allowing its assessment using fluorescent probes. The feature of this microfluidic design, requiring minimal handling and just a basic equipment, could facilitate the implementation of functional assays in hospitals to benefit cancer patients. Chapter 9 introduces kinetic detection of apoptosis events via caspase activation in a tumor-immune microenvironment on a chip, while Chap. 10 demonstrates how to establish a hypoxic 3-dimensional (3-D) tumor model using a cleanroom-free, micromilling-based microdevice and assesses the efficacy of the combinatorial treatment with CAR-T cells and PD-1/PD-L1 inhibition.

As it is well known, CAR-T cell therapy is revolutionizing the treatment of hematologic malignancies. However, there are still many challenges ahead before CAR-T cells can be used effectively to treat solid tumors and certain hematologic cancers, such as T-cell malignancies. Thus, several strategies are being investigated to improve the response to T cell therapies. For example, the functional fitness of CAR T cells directly correlates with their clinical efficacy. A standing limitation is the screening of approaches to improve cellular fitness in vivo, which is expensive and time-consuming. Chapter 11 describes a highly multiplexed single-cell secretomic assay based on the IsoLight platform to rapidly evaluate the impact of new pharmacologic or gene-engineering approaches aiming at improving CAR T cell function.

Further, several strategies are explored for immune cell engineering, from retroviral transduction to genome editing to knock-out or knock-in genes. Chapters 12, 13, 14, and 15 describe protocols for T cell engineering by using CRISPR-Cas9 technology, retroviruses, mRNA transfection, transposons, and lentiviruses respectively, while Chap. 16 describes procedures to evaluate human CAR T cells in solid tumors. Considering current concerns related to the control of transgene expression by engineered T cells such as cytokine release syndrome (CRS) and "on-target, off-tumor" cytotoxicity, we include in this book, Chapter 17 as an example of spatio-temporal control of T cell activity by nano-optogenetics. This system comprises synthetic light-sensitive CAR T cells and nanoparticles acting as in situ nano-transducer, allowing near-infrared light to wirelessly control CAR T cell immunotherapy.

Besides CAR T cells, engineering of cell-based therapy is further branching out to other immune cells such as natural killer cells (NK), among the first to infiltrate and fight tumors.

Current methods of producing large-scale CAR-NK cells mainly rely on mRNA transfection and viral vector transduction. However, mRNA CAR-NK cells were not stable in CAR expression while viral vector transduction mostly ended up with low efficiency. Chapter 18 describes an optimized protocol to generate CAR-NK cells by using the *piggyBac* transposon system via electroporation and to expand the engineered CAR-NK cells in large-scale together with artificial antigen-presenting feeder cells. This method can stably engineer human primary NK cells with high efficiency and supply sufficient scale of engineered CAR-NK cells for the future possible clinical applications.

Finally, we wanted to conclude with an example of what the integration of synthetic biology and immunology is further leading to. In Chap. 19, a novel approach for immunotherapy is presented based on bioengineered probiotics. Since probiotics have tumor-colonizing capabilities, a new probiotic *E. coli* Nissle 1917-based platform was developed, encoding a synchronized lysis mechanism for the localized and sustained release of blocking nanobodies against immune checkpoint molecules like programmed cell death protein-ligand 1 and cytotoxic T lymphocyte associated protein-4.

We believe that this comprehensive and detailed protocols will benefit bioengineers approaching to immunology and vice versa, creating a blossoming interdisciplinary community of synthetic immunologists.

Napoli, Italy ***Velia Siciliano***
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Chapter 1

Isolation of Live Immune Cells from the Tumor Microenvironment by FACS

Aikaterini Kafka, Christos Ermogenous, and Luigi Ombrato

Abstract

Isolation of live cells from the tumor microenvironment (TME) has represented a challenge, particularly from metastatic nodules that need to be identified within the entire metastatic tissue. Cherry-niche, an in vivo labelling technique, allows the isolation of all the different cell populations in the TME without needing to visually locate the metastatic cancer cell colonies. Therefore, neighboring TME cells can be isolated even from the early stages of cancer cell seeding and colonization in the metastatic tissue. Here, we show how to use Cherry-niche to identify and isolate neutrophils from the lung metastatic niche. We also provide examples of downstream analyses to characterize freshly isolated neutrophils ex vivo, such as Giemsa staining, reactive oxygen species (ROS) detection, and phagocytosis assays. Similar strategies can be used to isolate other immune and non-immune cells from the metastatic TME.

Key words Tumor microenvironment, Metastasis, Niche, Labelling, Cell isolation, Flow cytometry, Immune cells, Neutrophils

1 Introduction

Recent breakthroughs in cancer immunotherapies, such as immune checkpoint blockade (ICB), have led to dramatic clinical responses in patients by targeting the immune cells within the tumor microenvironment (TME) rather than the cancer cells themselves [1]. However, less than 20% of cancer patients respond to ICB [2–4]. The reasons why most patients do not respond to current immunotherapies are largely unknown. Given the critical role of immune cells in facilitating both pro- and anti-tumor responses [5–7], a better understanding of how immune cells interact with each other, as well as with cancer cells and stromal cells in the TME, will reveal new insights into how some immune cells acquire tumor-supportive functions. This knowledge will identify more specific

Aikaterini Kafka and Christos Ermogenous contributed equally to this work.

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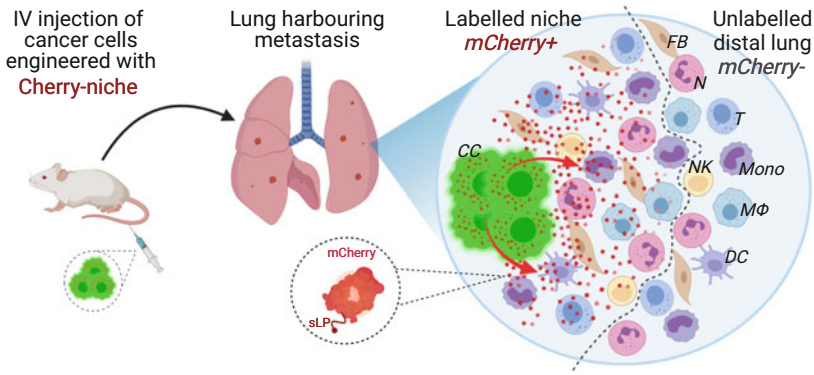

Fig. 1 Schematic illustration of the Cherry-niche labelling system. GFP$^+$ cancer cells are generated to express a modified mCherry protein (sLP-mCherry) that is made up of a lipid-soluble secretory peptide ('s') and a transactivator of transcription kappa (TATk) peptide ('LP'). The secreted sLP-mCherry can then be taken up by other TME cells within the surrounding tissue, such as immune cells, and is stored intracellularly in multivesicular bodies allowing for their detection and sorting by FACS. GFP allows to discriminate the labelling cancer cells from the labelled TME cells. Other markers instead of the GFP could also be used. CAF; cancer-associated fibroblast, CC; cancer cell, DC; dendritic cell, MΦ; macrophage, Mono; monocyte, N; neutrophil, NK; natural killer cell, T; T cell. (Image created using Biorender.com)

and effective targets, particularly in treating metastasis that remains largely incurable [8]. Nonetheless, the identification of the cells forming the metastatic TME, and their isolation for an in-depth characterization, has proven extremely challenging due to the technical difficulties of identifying a small number of cancer-associated cells within an otherwise healthy organ [9].

A recently developed in vivo labelling tool, Cherry-niche [10], allows the spatial identification and isolation of the cells that physically surround disseminated cancer cells (DCCs). Briefly, this system relies on using engineered tumor cells expressing a modified fluorescent protein (sLP-mCherry) that can be secreted and then taken up by surrounding cells within the tissue (Fig. 1). Once labelled, these cancer cell neighboring cells can be isolated using fluorescence-activated cell sorting (FACS) as live cells and used for ex vivo downstream applications. While a range of technologies have been developed in the last decade to isolate cells from the TME [9], Cherry-niche overcomes some of their key limitations. For example, it allows for unbiased identification of TME cells as no prior knowledge of the TME cells or their interactions is required. Therefore, it is possible to isolate all different cell types simultaneously, including less represented and/or unknown cellular components that make up the tumor "niche" [11].

Here, we present a protocol of how to use Cherry-niche labelling breast cancer cells to identify and isolate immune cells, namely, neutrophils, from the TME of lung metastases. In addition, we will provide examples of downstream analyses that can be applied to the

isolated immune cells to better understand the biological changes occurring specifically within metastatic niches. Importantly, Cherry-niche can be used to engineer tumor cells for a wide range of different cancer models to study the in vivo niche of interest and even be applied to non-cancer cells to extend its use beyond oncology research.

2 Materials

2.1 Cells

4T1 breast cancer cell line (ATCC, cat. no. CRL-2539; RRID: CVCL_0125) engineered using the sLP-mCherry plasmid (Ximbio, cat. no. 155083) as previously described [10, 11] (herein referred to as *labelling 4T1 cells*).

2.2 Reagents

DMEM (Thermo Fisher Scientific, cat. no. 41965-039)

Trypsin (0.25% [w/v]; Thermo Fisher Scientific, cat. no. 25050-014)

PBS (Gibco, cat. no. 10010015)

Hank's Balanced Salt Solution (HBSS, no calcium, no magnesium) (Thermo Fisher Scientific, cat. no. 14175–053)

Liberase TM (Roche, cat. no. 05401127001)

Liberase TH (Roche, cat. no. 05401151001)

DNase I (Sigma, cat. no. DN25-100 mg)

Red blood cell (RBC) lysis buffer (10X) (Miltenyi Biotec, cat. no. 130-094-183)

FCRγ blocking solution (10X) (Miltenyi Biotec, cat.no. 130-092-575)

Giemsa stain (C14H14ClN3S) (Sigma-Aldrich, cat.no. 51811-82-6)

Luminol-97% (C8H7N3O2) (Sigma-Aldrich, cat.no. 123072)

Peroxidase from horseradish (Sigma-Aldrich, cat.no. P8250)

PMA (phorbol 12-myristate 13-acetate) (C36H56O8) (Sigma-Aldrich, cat.no. P1585-1MG)

Phagocytosis Assay Kit (IgG FITC) (Cambridge Bioscience, cat.no. 500290-1ea-CAY)

Antibodies Used for Cell Sorting and Isolation of Neutrophils

CD45 APC-eFluor™ 780 (eBioscience, clone 30-F11, cat. no. 47–0451-82)

CD11b APC (Biolegend, clone M1/70, cat. no. 101212)

Ly6G V450 (BD biosciences, clone 1A8, cat. no. 560603). Alternatively the REAfinity Ly6G antibody can be used (Miltenyi Biotec, clone REA526, cat. no. 130-119-986).

2.3 Solutions

All solutions should be prepared in a sterile environment.

Culture Media

Mix DMEM with 10% (v/v) FBS (heat-inactivated) and 1% (v/v) penicillin–streptomycin**.**

Lung Digestion Solution

For each of the enzymes Liberase TM, Liberase TH, and DNase I, prepare a 5 mg/mL stock solution in HBSS (no calcium, no magnesium) and store them at −20 °C for up to 1 year.

For 5 mL of digestion solution, add 76 μL Liberase TM, 76 μL Liberase TH, and 25 μL DNase I in 4823 μL of HBSS.

1X RBC Lysis Buffer

For 10 mL 1X RBC lysis buffer, dilute 1 mL 10X RBC lysis buffer stock in 9 mL sterile deionized water.

FACS Buffer

For 500 mL FACS buffer, dissolve 2.5 g BSA and 372 mg EDTA in 500 mL of PBS (without $MgCl_2$ and $CaCl_2$). After the reagents have been dissolved, filter the FACS buffer through a 0.22-μm filter.

1X FCRγ Blocking Solution

For 500 μL 1X FCRγ blocking solution, dilute 50 μL 10X FCRγ blocking solution stock in 450 μL FACS buffer.

Luminol/HRP mix

In 1 mL HBSS, add 2 μL of luminol (50 mg/mL) and 1 μL of HRP (2 KU/mL).

Giemsa Stain

Dilute the Giemsa stain 1:20 in deionized water.

2.4 Equipment

T75 cell culture flasks (Corning, cat. no. 430641U)

Conical 50-mL and 15-mL tubes (Falcon, cat. nos. 352070 and 352096)

Eppendorf tubes (1.5 mL; Eppendorf, cat. no. 616201)

Pipettes (5, 10, and 25 mL; Falcon, cat. nos. 356543, 356551 and 356525)

Filter-tip pipettes (10, 20, 200, 1.000 μL; Starlab, cat. nos. S1120-3810, S1120-1810, S1120-8810 and S1122-1830)

CO_2 incubator (5% [v/v] CO_2, 37 °C; Eppendorf New Brunswick, model no. Galaxy 170R)

Syringes for intravenous injection (1 mL; Becton Dickinson, cat. no. 303172)

Sterile scissors and forceps (B Braun Medical, cat. nos. BD313R and BC061R)

Needle (27 gauge) (BD, cat. No. 302200)

Polystyrene FACS tubes (Falcon, cat no. 3352058)

Cell strainers (70 μm and 40 μm; Falcon, cat nos. 352350 and 352340)

Syringes (5 mL; BD Emerald, cat. no. 307731; 20 mL, BD Plastipak, cat. no. 300613)

Biosafety Level 2 (BSL2) cabinet (Clean Air, model no. CA/REV4)

Thermomixer-Mixer HC (Starlab, cat. no. S8012-0000)

Centrifuge (Eppendorf, model no. 5810R)

Flow cytometer (e.g., BD LSR Fortessa™ Cell Analyzer and BD FACSAria™ II cell sorter; BD Biosciences).

Epredia™ TPX single sample chamber, caps, and brown filter cards (Fisher scientific, cat. No. 11922355)

Adhesion slides SuperFrost Plus (VWR, cat. no. 631-0108)

Cytocentrifuge (Marshall Scientific, Shandon—Cytospin 3 Centrifuge)

NanoZoomer S60 slide scanner (OPTA-TECH)

Sterile 96-well white, flat-bottom, TC-treated microplate with clear lid (Corning, cat. no. 3917)

FLUOstar® Omega microplate reader (BMG Labtech)

3 Experimental Procedure

3.1 Metastatic Colonization in the Lung Using an In Vivo Mouse Model

1. Trypsinize and collect labelling 4T1 cells from T75 flask.
2. Count and resuspend cells in PBS at a final concentration of 1×10^7 cells per mL. Filter the cells by using a 40-μm cell strainer and keep them on ice.
3. Inject intravenously in the tail vein of 6- to 10-week-old BALB/c mice by using 100 μL of cell solution (1×10^6 labelling 4T1 cells) (*see* **Note 4.1.1**).
4. After 6 days, euthanize the mice and collect the metastatic lungs in ice-cold PBS (*see* **Note 4.1.2**).

3.2 Mouse Lung Tissue Processing to Obtain a Single Cell Suspension

1. Mince the lung tissue with scissors and a scalpel into a homogenous paste without visible clumps.
2. Transfer the minced tissue into a 1.5-mL Eppendorf tube and add 1 mL of lung digestion solution (*see* **Note 4.2.3**).
3. Incubate in the lung digestion solution for 30 min at 37 °C with shaking at 120 rpm in a Thermomixer-Mixer HC.

4. Collect the cell suspension, filter it through a 70-μm cell strainer, and then squeeze remaining cells through filter by using a 5-mL syringe plunger.
5. Add 4 mL of DMEM with 10% FBS on the filter to deactivate the digestive enzymes and spin at 300 g for 10 min at 4 °C.
6. Carefully remove the supernatant without touching the pellet. If the pellet still seems loose, spin the supernatant again for 5 min at 300 g and 4 °C and then remove the supernatant.
7. Resuspend the pellet in 5 ml of 1X RBC lysis buffer and filter it through a 40-μm cell strainer (*see* **Notes 4.2.3** and **4.2.4**).
8. Leave the red blood cell lysis buffer at RT for maximum 5 min and spin at 300 g for 10 min at 4 °C.
9. Resuspend the pellet in 5 ml of FACS buffer, filter it through a 40-μm cell strainer, and then squeeze the filter by using a 5-mL syringe plunger.
10. Spin at 300 g for 10 min at 4 °C and remove supernatant.

3.3 Staining Procedure to Identify and Isolate Neutrophils from the TME

1. Resuspend the pellet in 300 μL 1X FCRγ blocking solution and leave it at RT for 5 min (if using REAfinity antibodies from Miltenyi Biotec to stain cells, the incubation with FCRγ blocking solution is not required and cells should be resuspended in FACS buffer) (*see* **Note 4.3.5**).
2. Filter the sample with a 40-μm cell strainer and add the primary conjugated antibody mixture for neutrophil isolation at the concentration indicated by the manufacturer and incubate for 15–30 min on ice in the dark (e.g., Ly6G 1:150).
3. Add 2 mL of MACS buffer and spin at 300 g for 5 min at 4 °C and remove the supernatant (you can repeat the wash step optionally).
4. Resuspend the pellet in 300 μl of MACS buffer and add DAPI for 5 min.
5. Collect mCherry+ and mCherry− based on the gating strategy outlined in Fig. 2 using a cell sorter (e.g., BD FACSAria™ II) (*see* **Note 4.3.6**).

A similar strategy can be used to isolate other types of immune cells from the TME. A list of antibodies to identify the main immune cell populations is provided in Table 1.

3.4 Downstream Applications for Isolated Neutrophils (Ex Vivo)

After sorting, the isolated immune cells can be used in a variety of downstream applications including immune cell phenotyping, functional assays, and OMICs analyses (Fig. 3). Here, we provide examples of phenotypic and functional assays to characterize freshly sorted neutrophils (*see* **Notes 4.4.7** and **4.4.8**). Other assays may be appropriate for different immune cell types.

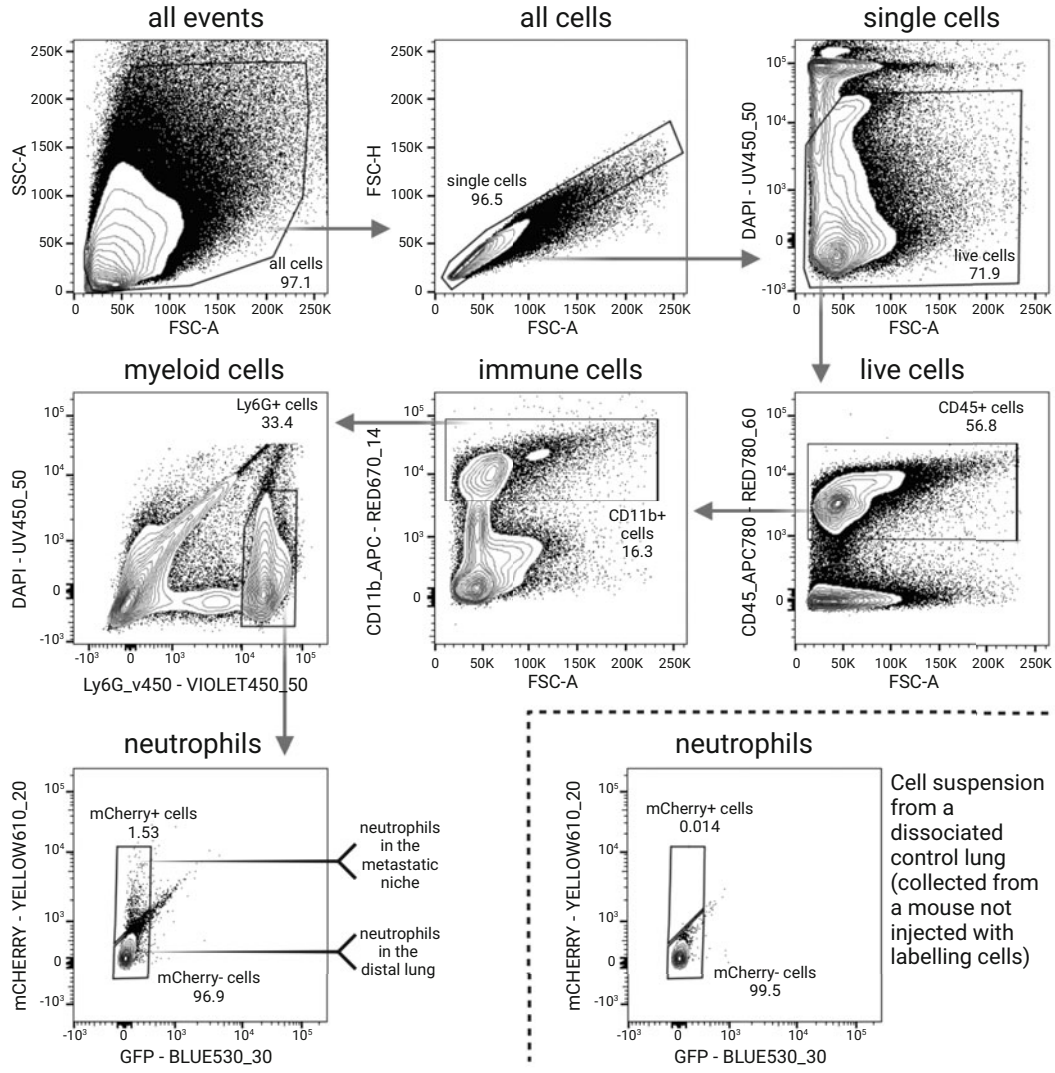

Fig. 2 Gating strategy for identifying neutrophils from the metastatic niche (mCherry$^+$) and the distal tissue (mCherry$^-$) from a metastatic lung. Arrows show gating hierarchy and gates have been drawn based on single staining or Fluorescence Minus One (FMO) controls. After gating on all events and single, live cells, CD45$^+$ immune cells, CD11b$^+$ myeloid cells, and Ly6G$^+$ neutrophils are gated sequentially. Then, mCherry$^+$ neutrophils from the niche can be discriminated from mCherry$^-$ neutrophils in the distal lung. We recommend plotting the cells in a mCherry-vs-GFP gate to exclude autofluorescent cells that are not "true" mCherry$^+$ cells (normally localized along the diagonal in this plot). In this experiment, BALB/c mice have been injected intravenously with labelling 4T1 cells and lungs collected at day 6 postinjection. For comparison, the bottom right plot shows a cell suspension from a control lung not injected with 4T1 labelling cells. FSC-A forward scatter area, SSC side scatter area, FSC-H forward scatter height

Neutrophils have a unique nuclear morphology that is linked to their maturation status [13]. Starting from common myeloid precursors that have a banded nucleus, neutrophils pass through several progenitor stages until they form mature neutrophils,

Table 1
Membrane markers and corresponding antibody clones for isolation of the most represented immune cell populations in mice

Cell population	Surface marker(s)	Antibody clone
Pan-immune cells	*$CD45^+$	30-F11
Neutrophils	**$CD11b^+$ **$Ly6G^+$**	M1/70 REA-526
Monocytes	**$CD11b^{high}$ **$Ly6C^{high}$** ($Ly6G^-$)	M1/70 HK1.4 (REA-526)
Macrophages	**$CD11b^+$ **F4/80+** ($Ly6G^-$)	M1/70 BM8 (REA-526)
Dendritic cells	**$CD11b^+$ **$CD11c^+$**	M1/70 N418
Cytotoxic T cells ($CD8^+$ T cells)	$CD3^+$ **$CD8^+$**	145-2C11 536.7
Helper T cells ($CD4^+$ T cells)	$CD3^+$ **$CD4^+$**	145-2C11 GK1.5
NK cells	***$CD49b^+$** or ***$NK1.1^+$** (and $CD3^-$)	DX5 or PK136 (145-2C11)

*CD45 is used as a pan-immune cell marker to discriminate hematopoietic cells ($CD45^+$) from other cells ($CD45^-$) in the tissue. **CD11b is used as pan-myeloid marker to discriminate myeloid ($CD11b^+$) and lymphoid immune cells ($CD11b^-$). ***CD49b is expressed by natural killer (NK) cells in most common inbred mouse strains (e.g., BALB/c, C57BL/6, C3H, CBA, DBA, NOD, AKR, SJL, and 129), whereas NK1.1 is only expressed by NK cells in CE, C57BL/6, NZB, C58, Ma/My, ST, SJL, and FVB/N mouse strain [12]

characterized by having multi-lobular nuclei with 2–5 lobes [13]. Giemsa stain is one of the most common histological stains used to label the cellular nucleus and distinguish the morphology and maturity stage of neutrophils (Fig. 3).

Neutrophils produce reactive oxygen species (ROS) which are important effector molecules in their cytotoxic functional response [14]. Below we describe how to measure total ROS production by neutrophils using a bioluminescent assay and neutrophil phagocytosis using a FACS-based fluorescently labelled bead assay (Fig. 3).

An in-depth characterization of neutrophils or other cells of interest from the TME can be achieved by using several OMICs techniques. OMICs analyses allow us to obtain large datasets of information at different levels, including RNA and protein levels, as well as epigenetic and metabolic profiles [15]. Single-cell RNA sequencing (scRNA-seq), for example, provides a quantitative measurement of the expression of every gene at the single-cell level that can help identify changes occurring specifically within the TME, by comparing $mCherry^+$ neutrophils from the metastatic niche with

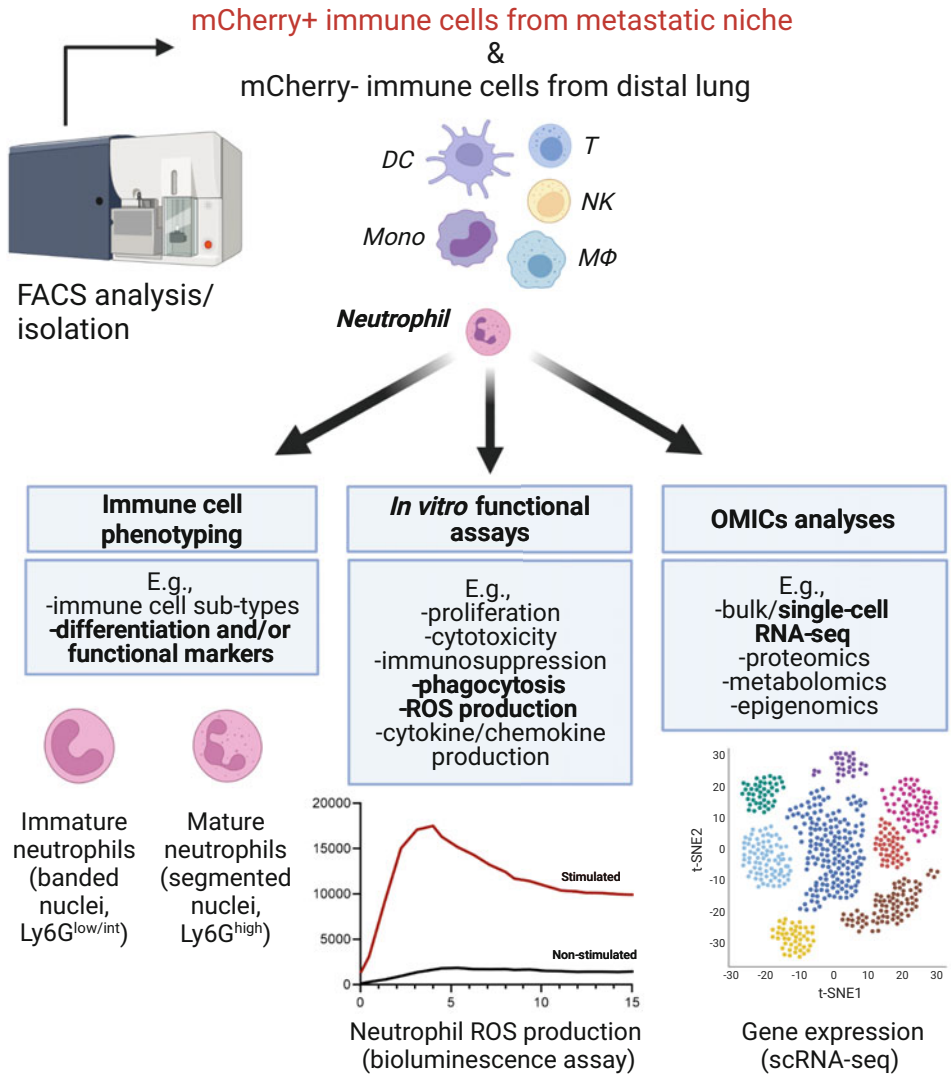

Fig. 3 Downstream ex vivo applications for freshly isolated neutrophils. Once neutrophils have been isolated by FACS, they can be characterized by using a variety of downstream analyses. Immune cell phenotyping by Giemsa staining allows identification of immature and mature neutrophils based on their nuclear morphology. Live immune cells can also be used in functional assays in vitro to test a variety of cellular functions. For instance, neutrophils can be stimulated and their total reactive oxygen species (ROS) production measured via bioluminescence. Finally, OMICs analyses, such as scRNA-seq, can generate large datasets to show cell specific changes in gene expression. DC dendritic cell, MΦ macrophage, Mono monocyte, N neutrophil, NK natural killer cell, T T cell. (Image created using Biorender.com)

mCherry$^-$ neutrophils from the distal tissue (Fig. 3). Moreover, different tools are now available to investigate intercellular communications by using scRNA-seq data, such as CellPhoneDB [16] and CellChat [17]. Such analyses can guide our understanding of how different populations of cells communicate at a molecular level in the metastatic niche and help us further explore both in vitro and in vivo the role of these molecular interactions.

3.4.1 Giemsa Staining

1. Add 50,000 neutrophils into the TPX chamber with filter card and spin onto adhesion slide at 800 rpm for 3 min in a Cytospin 3 Centrifuge.
2. Fix the slides in methanol for 5–7 min.
3. Place the slides in a chemical hood for 15 min to air-dry.
4. Stain the slides with Giemsa stain (diluted 1:20) for 45 min.
5. Wash with deionized water.
6. Air-dry and image the slides by using a scanner (e.g., NanoZoomer S60 slide scanner).

3.4.2 ROS Production Assay

1. Collect the neutrophils and count the cells with a hemocytometer.
2. Add the cells in HBSS in a 96-well plate (white plates are recommended for luminescence) and let them seed for 30 min at 37 °C.
3. Add 100 μl of luminol/HRP mix and centrifuge for 30 s at 100 g.
4. Add PMA (100 nM) to stimulate the neutrophils and start measuring the luminescence immediately and at regular intervals for 30 min in a plate reader.

3.4.3 Phagocytosis Assay

1. Plate 100 μl of neutrophils (1×10^4–10^5 cells) in a 96-well plate or in FACS tubes.
2. Add latex bead–rabbit IgG-FITC complex solution at a final dilution of 1:250 (*see* **Note 4.4.9**).
3. Incubate the samples at 37 °C for 1.5 h (the incubation time needs to be optimized for different immune cell types as phagocytic activity may vary).
4. Centrifuge the cells at 300 g for 5 min at RT and resuspend them in 400 μL of FACS buffer. If further surface staining (e.g., Ly6G) is required, add the antibodies at this step and incubate for 15–30 min (keep the cells on ice when you add the surface markers to avoid affecting bead binding).
5. Add DAPI for the live/dead staining and immediately analyze the samples with a flow cytometer (e.g., BD LSRFortessa™ Cell Analyzer) and gate for $FITC^+$ live neutrophils, which are the cells that have engulfed the FITC beads.

4 Notes

4.1 Metastatic Colonization in the Lung Using an In Vivo Mouse Model

1. The labelling 4T1 cell suspension must be filtered before the intravenous injections to remove any cell aggregates as they can obstruct vessels and cause immediate mouse death. As with most epithelial cell lines, 4T1 cells tend to aggregate; therefore,

it is recommended to refilter the cell suspension immediately before injecting mice.

2. In this protocol, we euthanize the mice and collect the lungs on day 7 after intravenous injection. At this stage we expect to find micrometastases in the lungs. Different time points need to be used to study other metastatic stages. We suggest collecting the lungs at day 3 postinjection to identify the TME at the stage of metastatic colonization and at day 10 for macrometastases. The timeframe for metastatic colonization and growth in the lungs after intravenous injection is dependent on the specific cancer cell line; therefore, collection times may need to be adjusted.

4.2 Mouse Lung Tissue Processing to Obtain a Single-Cell Suspension

3. The digestion solution and the 1X RBC lysis buffer should be prepared fresh each time.
4. Do not exceed the 5-min incubation with 1X RBC lysis buffer. This buffer contains ammonium chloride to lyse the red blood cells. However, incubation times longer than 5 min can also affect viability of other cells, such as lymphocytes.

4.3 Staining Procedure to Identify and Isolate Neutrophils from the TME

5. If REAfinity antibodies from Miltenyi Biotec are used, the step with FCRγ blocking is not required. We recommend performing the step with the FCRγ blocking solution when a combination of antibodies including REAfinity and non-REAfinity antibodies is used within the same mix.
6. Neutrophils are very fragile and can easily be damaged during the sorting process as they enter the collection tube under high pressure causing cell death/loss. Therefore, we recommend using a 100-μm nozzle for FACS to reduce the pressure and speed at which cells enter the collection tube.

4.4 Downstream Applications for Isolated Neutrophils (Ex Vivo)

7. Neutrophils have a short life span in vitro, estimated to be less than 24 h. We recommend that all the functional ex vivo assays be performed within the first 24 h, when possible.
8. For neutrophil characterization or any functional assays that require flow cytometry and overnight incubation periods, neutrophils should be re-stained with the same antibody as some cells may lose their antibody staining after 24 h.
9. For the phagocytosis assay, a 1:100 to 1:500 final dilution range is recommended for the latex bead–rabbit IgG-FITC complex solution; however, this may require optimization when adding beads to different immune cell types.

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Chapter 2

In Vitro Evaluation of Cancer Cell Immunogenicity and Antigen-Specific T-Cell Cytotoxicity by Flow Cytometry

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Abstract

A cardinal principle of oncoimmunology is that cancer cells can be eliminated by tumor-infiltrating cytotoxic CD8 T lymphocytes. This has been widely demonstrated during the last 20 years and also recently harnessed for therapy. However, emerging evidence indicates that even neoplasms showing striking initial responses to conventional and targeted (immuno)therapies often acquire resistance, resulting in tumor relapse, increased aggressiveness, and metastatization. Indeed, tumors are complex ecosystems whose malignant and nonmalignant cells, constituting the tumor microenvironment, constantly interact and evolve in space and time. Together with patient's own genetic factors, such environmental interplays may curtail antitumor immune responses leading to cancer immune evasion and natural/acquired (immuno)-therapy resistance. In this context, cancer stem cells (CSCs) are thought to be the roots of therapy failure. Flow cytometry is a powerful technology that finds extensive applications in cancer biology. It offers several unique advantages as it allows the rapid, quantitative, and multiparametric analysis of cell populations or functions at the single-cell level. In this chapter, we discuss a two-color flow cytometric protocol to evaluate cancer cell immunogenicity by analyzing the proliferative and tumor-killing potential of ovalbumin (OVA)-specific CD8 OT-1 T cells exposed to OVA-expressing MCA205 sarcoma cells and their CSC counterparts.

Key words Tumor microenvironment, T cells, Cancer immunogenicity, Immune escape, Flow cytometry

Abbreviations

Ag	antigen
APC	*allophycocyanin*
BM	bone marrow
CFSE	*5(6)-carboxyfluorescein diacetate N-succinimidyl ester*
CSC	cancer stem cell
DAPI	4′,6-diamidino-2-phenylindole
DC	dendritic cell

Authors Eliana Ruggiero and Antonella Sistigu share senior co-authorship to this chapter.

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EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FSC-A	forward scatter area
GM-CSF	granulocyte–macrophage colony-stimulating factor
IL	interleukin
MCA205-OVA	ovalbumin-expressing MCA205
MHC-I	major histocompatibility complex I
MHC-II	major histocompatibility complex class II
OVA	ovalbumin
PB	Pacific Blue
PBS	phosphate-buffered saline
PI	*propidium iodide*
RBC	red blood cell
RPMI	Roswell Park Memorial Institute
RT	room temperature
SSC-A	side scatter area
SSC-W	side scatter width
TCR	T cell receptor
TGFβ	transforming growth factor beta
TME	tumor microenvironment
TNFα	tumor necrosis factor alpha

1 Introduction

The biological implications of the tumor microenvironment (TME) on cancer onset, progression, and response to therapy have drawn growing attention over the past few years [1]. Immune cells are key components of the TME and critically affect tumor-host coevolution. Indeed, cancer cells need to constantly cope with immune surveillance in a process known as the "cancer immunity cycle," whose main, but not sole, players are represented by dendritic cells (DCs) and cytotoxic CD8 T lymphocytes [2, 3]. In the most simplified version of this stepwise process, DCs initiate primary immune responses at peripheral sites by capturing and processing either tumor-associated antigens (Ags) and tumor-specific Ags (also known as neo-Ags) which are then loaded on major histocompatibility complex class I (MHC-I) molecules [4]. Hence, such mature DCs traffic to lymphoid organs where they cross-prime and activate tumor-specific naïve CD8 T cells by also providing co-stimulatory signals including, but not limited to, the upregulation of CD80/CD86 molecules and the secretion of proinflammatory cytokines (e.g., interleukin [IL]-1β, IL-12, and tumor necrosis factor alpha [TNFα]) [5, 6]. Once activated, effector CD8 T lymphocytes undergo a clonal expansion, migrate toward and infiltrate the tumor stroma where they specifically recognize target cancer

cells through their T cell receptor (TCR) repertoire, differentiate into effector cells in order to exert their cytotoxic activity, and finally, enter into an apoptosis-related contraction phase which spares a pool of long-lived tumor Ag-specific memory cells [7–9]. The goal of cancer immunotherapy is to (re)instate and boost this self-sustaining cycle and, at the same time, to block "corrupted" immune elements promoting tumor initiation and propagation. However, clinical data indicate that in cancer patients the "cancer immunity cycle" does not perform optimally, leading to primary or acquired (immuno)therapy resistance [10, 11].

The progressive acquisition of (epi)genetic dysregulation patterns, as well as the dynamic interplay with TME components, enables cancer cells to tackle the constant evolutionary pressure to which they are subjected and thus to overcome the nutritional, trofic, and immunological barriers to their proliferation and survival [12–14]. It is now well established that the major mechanisms underlying the success of anticancer immunity include the capability of T cells to efficiently home into the tumor bed and once here to unleash a robust and persistent immune response. In this regard, depending on the immune contexture, tumors are distinguished into three main immune profiles and specifically: (1) "inflamed" or "hot" tumors, which display high T cell infiltration in malignant cell nests and so are prone to respond to immunotherapy; (2) "immune-excluded" tumors, which seclude immune cells at the tumor margin and so are generally associated with poor disease outcome and resistance to immunotherapy; and finally, (3) the so-called "immune-desert" or "cold" tumors, which are characterized by a scarce T cell immune infiltration and reduced sensitivity to immunotherapy in a variety of clinical settings [15–18]**.** If it is true that tumors continuously evolve to cope with microenvironmental variations, at the same time the TME constantly shapes tumor architecture and composition thus driving tumor Ag evolution and cancer immune evasion [19]. This generally occurs through the selection of poor immunogenic cancer cell variants (immunoediting) [20, 21] or the establishment of an immunosuppressive TME (immune subversion) [22] where cancer cells contrast the function of effector CD8 T lymphocytes by expressing immune checkpoint ligands [23], recruiting regulatory T and myeloid-derived suppressor cell populations [24, 25], and secreting suppressive cytokines (e.g., IL-10, transforming growth factor beta [TGFβ]) and metabolites (e.g., adenosine, prostaglandins, lactate, kynurenine) [26].

This scenario of immunosuppression provides a fertile soil for cancer stem cells (CSCs), the immature, tumorigenic, and poorly immunogenic cell subset within the tumor bulk capable of self-renewal and multilineage differentiation and responsible for tumor initiation, progression, metastasis, and therapy resistance [27]. Given their immune-privileged nature, CSCs not only evade

immune attacks by cell-autonomous tricks, but also actively suppress immune responses by remodeling the immunological component of the TME, which in turns drives CSC (re)generation and expansion [28]. In this regard, we recently identified a hitherto unknown epigenetic mechanism of tumor evolution by which type I interferon signaling elicited during immunogenic chemotherapy promotes cancer cell stemness, acquired resistance, and immune evasion [29].

Flow cytometry is an essential tool for studying the TME. By simultaneously analyzing multiple physical and fluorescent parameters, this technique offers the possibility to quickly identify and quantify at single-cell resolution different cell populations and functions from heterogeneous and even heterotypic cell suspensions [30]. Compared to other single-cell analytical technologies, flow cytometry finds wide application in both experimental and clinical settings for its relatively lower sample volume requirements along with shorter and easier sample preparation and instrument setup protocols [31]. In addition when equipped with fluorescence-activated cell sorting (FACS) capability, flow cytometry enables the isolation of distinct cell subsets which can be further characterized by downstream in vitro and in vivo assays [32].

Here we describe a detailed flow cytometric protocol to assess cancer cell immunogenicity and Ag T cell-specific cytotoxicity focusing on the analysis of the proliferation rate and tumor-killing potential of CD8 H-2K^b/ovalbumin (OVA)-specific OT-1 T cells exposed to OVA-expressing MCA205 sarcoma cells (MCA205-OVA) and their CSC counterparts (CSC-OVA).

2 Materials

2.1 Disposable

- 75-cm^2 cell culture treated flasks (Corning®, New York, NY).
- 100-mm Petri dishes (Nunc™).
- 24-well plates (Corning®).
- 40-μm cell strainer (Falcon, Corning®).
- 70-μm cell strainer (Falcon, Corning®).
- Cell counting slides.
- 15- and 50-mL tubes (Falcon, Corning®).
- 5-mL polystyrene tubes (Falcon, Corning®).
- 1.5-mL Eppendorf microtubes (Eppendorf®, Hamburg, Germany).
- Pipette aid and serological pipettes (2-mL, 5-mL, 10-mL).
- Pipettes and sterile tips (1–10-μL, 20–200-μL, 1000-μL).
- Multichannel pipette (20–200-μL).

- Insulin syringes (BD Veo™ insulin syringes with BD Ultra-Fine™ 6-mm × 31G needle, Becton, Dickinson and Company, Franklin Lakes, NJ USA).
- 1-mL sterile syringe (BD PlastipakTM, Becton Dickinson [BD, Franklin Lakes, NJ).
- 10-mL sterile syringe (BD PlastipakTM).
- 27-gauge sterile needle (BD PlastipakTM).
- MACS separation columns (LS, MS Miltenyi Biotec).
- MACS Separators (MiniMACS™, MidiMACS™ Miltenyi Biotec).
- MACS Multistand (Miltenyi Biotec).
- Aluminum foil.
- Ice.
- Liquid nitrogen.
- Timer.
- Sterile scalpels, surgical forceps, scissors, and pliers.
- Adsorbent wipes.

2.2 Equipment

- Microbiologically controlled animal facility equipped with Class II safety cabinet.
- Humidified cell culture incubator at 37 °C with 5% CO_2.
- Laboratory safety cabinet (Class II).
- Laboratory bench.
- Optical microscope.
- Refrigerable centrifuge.
- Laboratory refrigerator (4 °C).
- Ice machine.
- Thermostatic bath.
- Flow cytometer (such as CytoFLEX, with up to three lasers: blue [488-nm], red [633-nm], and violet [405-nm] Beckman Coulter s.r.l., Brea, CA).
- Software for flow cytometry analysis (such as FlowJo software [FlowJo, LLC, Becton, Dickinson and Company, Franklin Lakes, NJ USA]).
- Software for statistical analysis (such as Prism GraphPad).

2.3 Reagents

- MCA205-OVA murine fibrosarcoma cells.
- Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin G sodium salt, 100 μg mL^{-1} streptomycin sulfate (all from Gibco, Thermo

Fisher, Carlsbad, CA) and 50 μg mL^{-1} hygromycin (Invitrogen, Thermo Fisher).

- Phosphate-buffered saline (PBS, Gibco-Thermo Fisher).
- Trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Gibco-Thermo Fisher).
- Spleens from transgenic histocompatible C57BL/6-Tg (TcraTcrb)1100Mjb/Crl OT1 (H-2^b) mice (Charles River, Wilmington, Massachusetts, USA).
- Bone marrows (BM) from histocompatible (H-$2K^b$) C57BL/6 J mice (Charles River).
- Red blood cell (RBC) lysis buffer (eBioscience™, Thermo Fisher).
- EDTA, 0.1 M, pH 7.2 (Sigma-Aldrich, Saint-Louis, MO).
- Separation buffer (Miltenyi Biotec).
- CD8a (Ly-2) MicroBeads (Miltenyi Biotec).
- Murine recombinant interleukin 4 (IL-4, Miltenyi Biotec).
- Murine recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF, Miltenyi Biotec).
- SIINFEKL peptide (Sigma-Aldrich).
- 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma-Aldrich).
- 1% FBS in cold PBS.
- Anti-mouse allophycocyanin (APC)-conjugated CD8a (clone 53–6.7, eBioscience™, Thermo Fisher) and IgG isotype control (eBioscience™, Thermo Fisher).
- Anti-mouse Pacific Blue (PB)-conjugated CD45 (clone 30-F11, eBioscience™, Thermo Fisher) and IgG isotype control (eBioscience™, Thermo Fisher).
- 4′,6-Diamidino-2-phenylindole (DAPI, Thermo Fisher).
- Propidium iodide (PI, Thermo Fisher).
- 70% ethanol (v/v in sterile distilled water).

3 Methods

3.1 Cell Culture Routine

1. Murine fibrosarcoma cells stably expressing the OVA Ag (MCA205-OVA) (*see* **Note 1**) and their CSC-OVA counterparts, derived as in ref. [29], are routinely maintained in a humidified cell culture incubator under standard culture conditions (37 °C, 5% CO_2) in Roswell Park Memorial Institute (RPMI) 1640 growth medium plus 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU mL^{-1} penicillin G

sodium salt, 100 μg mL^{-1} streptomycin sulfate, and 50 μg mL^{-1} hygromycin (complete growth medium) (*see* **Note 2**).

2. After thawing, cells need ~one-week recovery time to adapt to optimal culture conditions and reestablish normal cell cycles.
3. A density of 5 × 10^6 cells in 15-mL of complete growth medium in 75-cm^2 flasks is the optimal culture condition (*see* **Note 3**).
4. At a 75–80% maximum confluency (*see* **Note 4**), complete growth medium is discarded; the cell monolayer is washed with pre-warmed phosphate-buffered saline (PBS) to completely remove FBS (*see* **Note 5**) and then detached by 1- to 2-min incubation at 37 °C with 1.5-mL of pre-warmed 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) (*see* **Notes 6–8**).
5. Eight- to 10-mL of pre-warmed complete growth medium is then added to detached cells and the cell suspension is collected into a 15-mL tube. Cells are hence washed for 5-min at 1200-rpm at room temperature (RT) and reseeded in complete growth medium either upon dilution for maintenance culture (*see* **Note 9**) or upon counting for experimental procedures as detailed in Subheading 3.4.

3.2 Bone Marrow (BM) Processing and DC Differentiation (See Note 10)

1. Femurs and tibias previously explanted from immunocompetent mice (in our case histocompatible [H-$2K^b$] naïve C57Bl/6 J) are placed on ice in a 100-mm non-tissue culture-treated Petri dish filled with PBS and, while grasping the bone with forceps, the epiphyses are removed on both sides of the bone with scissors (*see* **Notes 11** and **12**).
2. A 1-mL syringe with a 27-gauge needle is filled with cold complete growth medium and the tip of the needle is gently put through the bones, while grasping it with forceps.
3. The marrows are eluted in a new 100-mm Petri dish containing complete growth medium by firmly injecting and pushing the medium into the bones using the prefilled syringe (*see* **Note 13**).
4. BMs from all four leg bones are pooled into a single Petri dish and cell clumps dissolved by gently pulling and releasing the media and marrows into a syringe.
5. BM-derived cell suspensions are placed into a 70-μm cell strainer on the top of a 50-mL tube, eluted with complete RPMI 1640 medium (*see* **Note 14**) and then washed twice in 10–15-mL of complete growth medium at 1500-rpm for 5-min at 4 °C.
6. One-mL of ice-cold red blood cell (RBC) lysis buffer is added to the cell pellet and incubated for 1- to 3-min at RT.

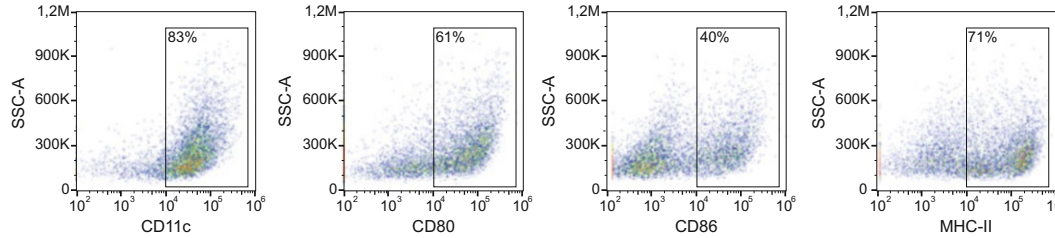

Fig. 1 Flow cytometry analysis of differentiated DCs. DCs are differentiated from BM-isolated precursor cells kept in culture for 10-days in the presence of 10 ng mL^{-1} of GM-CSF and IL-4. Representative monoparametric plots show the percentage of CD11c, CD80, CD86, and MHC-II positive DCs previously gated on morphology, singlets, and viability

7. Thereafter, cell suspension is washed twice as in **step 5**, and the resulting RBC-free white pellet is resuspended with 10-mL of complete growth medium and placed on ice prior cell count (*see* **Note 15**).
8. Upon cell count, BM-derived cells are resuspended at 2×10^6 *per* mL in complete growth medium further supplemented with 10-ng mL^{-1} of granulocyte–macrophage colony-stimulating factor (GM-CSF) and 10-ng mL^{-1} of IL-4 and 2-mL per well of this cell suspension is seeded in non-tissue culture-treated 6-well plates (day 0) (*see* **Note 16**).
9. On days 2,4,6, and 8 cells are gently washed with pre-warmed PBS and supplied with 1-mL *per* well of new complete growth medium to which 10 ng mL^{-1} of GM-CSF and IL-4 are **freshly added (*see* Note 17).**
10. On day 10, floating and lightly adherent DCs in the culture (*see* **Note 18**) are collected in 50-mL tubes and then washed for 5-min at 1200-rpm at RT in complete growth medium before cell count.
11. At this stage, it is recommended to verify that the differentiation protocol has efficiently worked by checking BM-derived DCs for the surface expression of CD11c, CD80, CD86, and MHC class II (MHC-II) molecules as illustrated in Fig. 1.

3.3 Spleen Processing and CD8 OT-1 T Cell Isolation

1. Spleens are explanted from transgenic immunocompetent C57BL/6-Tg(TcraTcrb)1100Mjb/J OT-1 mice (*see* **Note 19**), placed into 40-μm cell strainer atop a 50-mL tube, and mechanically smashed with the plunger of a 10-mL syringe.
2. Upon cell strainer rinsing with 20-mL of complete growth medium, the obtained splenocyte cell suspension is centrifuged twice at 1500-rpm for 5-min at RT in 10-mL of complete growth medium.

3. Cell pellet, containing approximately 1×10^8 cells, is then resuspended in RBC lysis buffer (refer to Subheading 3.2, **step 6**) to improve leukocyte enrichment.
4. After washing the splenocytes twice (as in **step 2**), the resulting RBC-free white pellet is resuspended in 10-mL of complete growth medium and then centrifuged at 800-rpm for 10-min at RT in order to remove platelets.
5. The obtained cell pellet is hence resuspended in 40-μL of cold separation buffer (*see* **Note 20**) with 10-μL of Biotin-Antibody Cocktail *per* 10^7 total cells (*see* **Note 21**).
6. Upon incubation for 5-min at 4 °C, 30-μL of separation buffer and 20-μL of Anti-Biotin MicroBeads are added and samples are further incubated for 10-min at 4 °C.
7. Immediately after, samples are transferred into MACS separation columns (*see* **Note 22**) to subsequent magnetic cell separation which occurs for negative selection.
8. Columns are placed in the magnetic field of a suitable MACS Separator and rinsed with 3-mL of separation buffer.
9. Cell suspension is transferred into the column and the eluate, representing the fraction of unlabeled cells enriched in CD8 OT-1 T cells, is collected in a 15-mL tube.
10. To increase the CD8 OT-1 T cell yield, columns are washed again with 3-mL of separation buffer and the collected unlabeled cells that pass through are combined with the flow-through from **step 9** (*see* **Note 23**).
11. Isolated CD8 T cells are washed for 5-min at 1500 rpm at RT in complete growth medium before cell count and CD8 OT-1 fraction is then checked for purity by flow cytometry using a CytoFLEX (Beckman Coulter).

3.4 CD8 OT-1 T Cell Cross-Priming and Activation.

1. Two $\times 10^6$ MCA205-OVA and CSC-OVA cells are plated in a 100-mm tissue culture-treated Petri dish in 10-mL of complete growth medium, UV irradiated at a 9-cm distance for 3-min as in ref. [33], and then incubated at 37 °C, 5% CO_2 for at least 6- h to ensure the induction of cancer cell death (***see*** **Note 24**).
2. Thereafter, UV-irradiated apoptotic cells are washed for 5-min at 1200-rpm at RT and co-cultured with BM-derived DCs, isolated as described in Subheading 3.2, at a 2:1 ratio (6×10^5 apoptotic MCA205-OVA or CSC-OVA cells for 3×10^5 DCs) in 24-w plates in 1-mL of fresh complete growth medium for 24-h (*see* **Notes 25** and **26**) at 37 °C.
3. BM-derived DCs that had previously taken up apoptotic MCA205-OVA or CSC-OVA cells are then cultured with splenic CD8 OT-1 T cells, purified as in Subheading 3.3, and resuspended in 500-μL of fresh complete growth medium, at a

5:1 ratio (3×10^5 DCs for 6×10^4 CD8 OT-1 T cells) for 72-h in a final volume of 1.5-mL.

4. After 72-h, cross-primed CD8 OT-1 T cells are hence counted, centrifuged for 5-min at 1500-rpm at RT, resuspended in PBS supplemented with 1% (v/v) FBS at 1×10^7 cells *per* mL, and then labelled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) dye at a final concentration of 1-μM for 10-min at 37 °C in the dark (*see* **Note 27**).
5. Upon incubation, cells are washed twice with 10-mL of PBS supplemented with 1% (v/v) FBS prior to centrifugation at 1500-rpm for 5-min at 4 °C.
6. CFSE-stained CD8 OT-1 T cells are then restimulated with live MCA205-OVA or CSC-OVA cells at 1:5 ratio (6×10^4 CD8 OT-1 T cells for 3×10^5 MCA205-OVA or CSC-OVA cells) for 72-h in 24-w plates in 1.5 mL of fresh complete RPMI 1640 medium.

3.5 CD8 OT-1 T Cell Proliferation Analysis by Flow Cytometry

1. Three-days later, cells are recovered and centrifuged for 5-min at 1500-rpm at RT.
2. For cell surface staining, cells are resuspended in 100-μL of 1% FBS in cold PBS containing 0.25-μg of either IgG isotype control or anti-mouse allophycocyanin (APC)-conjugated CD8a (*see* **Note 28**).
3. Cells are incubated for 20-min at 4 °C in the dark (*see* **Note 27**) and washed twice in 1% FBS in cold PBS. The vitality dye 4′,6-diamidino-2-phenylindole (DAPI) is added at a final concentration of 1-μM (*see* **Note 29**) prior to flow cytometric analysis of CD8 and CFSE levels by means of CytoFLEX (Beckman Coulter).
4. Data analysis is performed using the FlowJo software.
5. During acquisition and analysis, samples are sequentially gated for the following: morphology, by plotting forward scatter area (FSC-A, x-axis) and side scatter area (SSC-A, y-axis) (gate 1); singlets, by plotting SSC-A (x-axis) and side scatter width (SSC-W, y-axis) (gate 2); viability, by plotting blue laser area (emission 461-nm, x-axis) and SSC-A (y-axis) (*see* **Note 30**) (gate 3); and finally CD8 positivity, by plotting red laser area (emission 657-nm, x-axis) and SSC-A (y-axis) (gate 4). On gate 4, cells are analyzed for CFSE levels, by plotting green laser area (emission 521-nm, x-axis) and the number of events (cell count, y-axis) (*see* **Note 31**) (Fig. 2a).

3.6 Cancer Cell Killing Analysis by Flow Cytometry

1. After 72-h of incubation (refer to Subheading 3.4, **step 5**), cells are recovered and centrifuged for 5-min at 1500-rpm at RT.

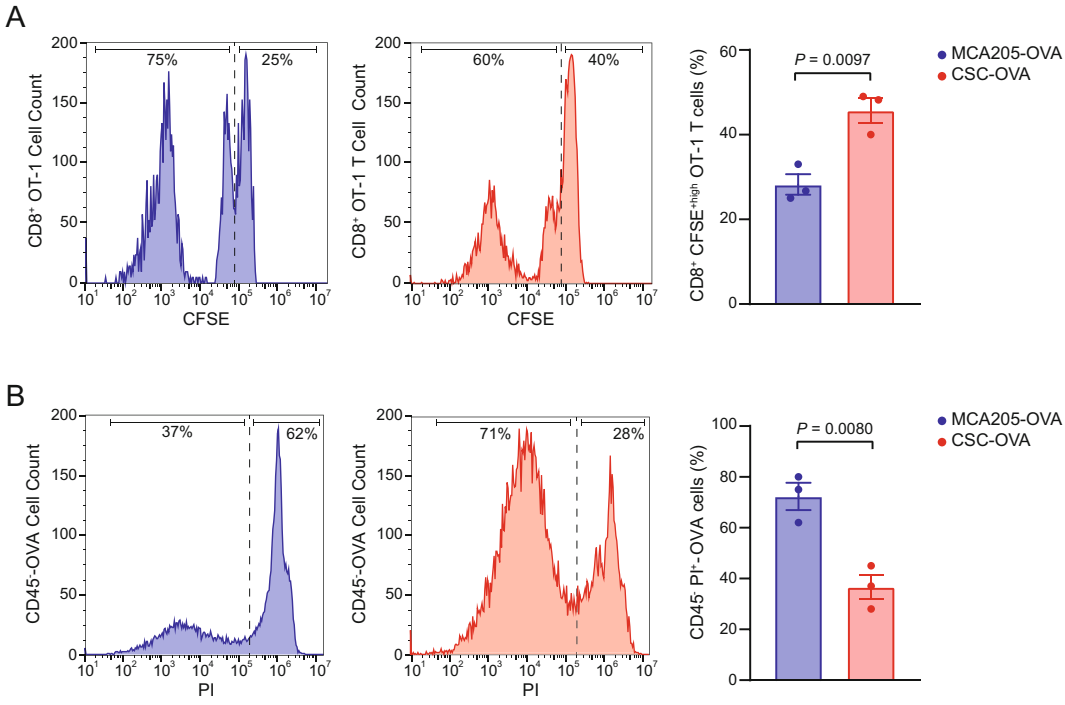

Fig. 2 Flow cytometry analysis of the proliferative and tumor-killing potential of OVA-specific CD8 OT-1 T cells. (**a**) CD8 OT-1 T cells, previously primed with BM-derived DCs that had taken up apoptotic MCA205-OVA and their CSC counterparts and then boosted with viable cells of the same type, have been analyzed for their proliferation rate by assessing CFSE levels on live-gated $CD8^+$ cells. The histograms represent the percentage (mean ± SEM; $n = 3$) of not-proliferating $CFSE^{+high}$ CD8 OT-1 T cells. (**b**) In parallel, $CD45^-$ MCA205-OVA and $CD45^-$ CSC-OVA cells have been analyzed for their ability to resist CD8 OT-1-mediated killing by assessing PI levels on $CD45^-$ cells. The histograms represent the percentage (mean ± SEM; $n = 3$) of dying PI^+CD45^- cells

2. Cells are resuspended in 100-µL of 1% FBS in cold PBS and surface stained with 0.125-µg of either IgG isotype control or anti-mouse Pacific Blue (PB)-conjugated CD45 (*see* **Note 28**).
3. Upon incubation for 20-min at 4 °C in the dark to avoid the loss of signal due to photobleaching of fluorochromes, cells are washed twice in 1% FBS in cold PBS.
4. The vitality dye propidium iodide (PI) is added at a final concentration of 1-µM (*see* **Note 29**) prior to flow cytometric analysis.
5. Samples are assessed for CD45 and PI levels using a CytoFLEX (Beckman Coulter) and data are analyzed by using FlowJo software v10.0.7.
6. During acquisition and analysis, samples are sequentially gated for the following: morphology, by plotting FSC-A (x-axis) and SSC-A (y-axis) (gate 1); singlets, by plotting SSC-A (x-axis) and SSC-W (y-axis) (gate 2); CD45 positivity, by plotting blue

laser area (emission 455-nm, x-axis) and SSC-A (y-axis) (gate 3); and finally cells are analyzed for PI, by plotting red laser area (emission 617-nm, x-axis) and the number of events (cell count, y-axis) (see **Note 31**) (Fig. 2b).

4 Remarks

In this chapter, we describe a detailed feasible and reliable protocol to gain insight into cancer cell immunogenicity by taking advantage of flow cytometry technology. With the aim to in vitro reproduce the "cancer immunity cycle," we first isolated BM precursor cells from histocompatible immunocompetent mice and then we induced them to differentiate into DCs. As shown in the plots in Fig. 1, this approach ensures a high yield of differentiated BM-derived DCs expressing the surface molecules CD11c, CD80, CD86, and MHC-II. As a following step, we promote DC-mediated uptake of apoptotic bodies, and thus tumor Ag processing and loading on MHC-I, by putting BM-derived DCs in co-culture with previously UV-irradiated apoptotic MCA205-OVA cells and their CSC-OVA counterparts. Thereafter, OVA-specific CD8 OT-1 cells were added to the system, cross-primed by BM-derived DCs, and then boosted with viable cancer cells of the same type. We then investigated the proliferative and cancer-killing potential of such activated CD8 OT-1 T cells. Specifically, to assess T cell proliferation we made use of the CFSE tracker in combination with the APC-conjugated anti-CD8a antibody. Notably, we choose CFSE for its easy and efficient labelling with an emission wavelength well separated from the APC-conjugated anti-CD8a antibody (CFSE, 521-nm; APC, 657-nm). By exploiting this approach, we demonstrated that CD8 OT-1 T cells challenged with CSC-OVA cells exhibited a significantly lower expansion than MCA205-OVA counterparts (Fig. 2a). Additionally, the cytotoxicity of OVA-specific CD8 OT-1 T cell was indirectly analyzed by evaluating cell death levels with PI on the $CD45^{-}$ cancer cell fraction. In line with the immune privileged nature ascribed to CSCs, we showed that CSC-OVA cells resisted to CD8 OT-1 T-mediated killing (Fig. 2b).

This in vitro approach may be easily adapted for different experimental conditions and requirements. However, we are aware that cancer cell immunogenicity is a wide and intricate field of study and that the protocol we propose points out only few aspects. Therefore, we strongly recommend to further confirm and extend experimental results by performing additional in vitro and in vivo assays to explore T cell cytokine production, chemotaxis, activation, and dysfunctional states.

5 Notes

1. A detailed method on how to efficiently transfect MCA205 cells with the OVA Ag is described in [34, 35]. Any other cell line expressing a specific antigen can be used.
2. Culture conditions should be adapted according to the cell line of choice.
3. The number of seeded cells may vary across different cell lines usually depending on the cell size and proliferation rate.
4. Cell confluence dramatically influences the cell state. If cells are little or too confluent, metabolic perturbations occur which can favor proliferative arrest and clonal selection or cell stress by starvation and death, respectively. MCA205-OVA cells should be passaged at 1:20 ratio twice *per* week and this should be adapted in function of each specific cell line doubling time. Otherwise, if the optimal confluence is not reached, it is recommended not to detach cells but only renew the medium until proper confluence is achieved.
5. FBS contains protease inhibitors that may inactivate trypsin enzymatic activity.
6. Optimal activity of trypsin is achieved at 37 °C; hence, pre-warmed trypsin accelerates cell detachment.
7. Trypsin incubation time differs depending on the cell type. For MCA205-OVA cells 2-min is enough to obtain a complete cell detachment. As a general guideline, long-term incubation with high trypsin concentration should be avoided as it may stripe cell surface proteins and trigger cell death.
8. At this step, cell detachment state should be checked by a light microscope.
9. The cell passage number is an important parameter to be aware of because it affects cell line's characteristics over time. It should not be too forward because the more cancer cells are in culture, the further is the random mutational burden they can accumulate which, in turn, may lead to the expression of aberrant phenotypes.
10. All the reagents and samples must be kept on ice during the entire procedure. A sterile technique is required for the isolation of BM cells and all tools should be rinsed with 70% ethanol.
11. As described in detail in [36], just after euthanization the mice abdomen is placed down-faced on a towel sprayed with 70% ethanol and by using scissors the skin is cut at the back to expose the legs. The hind legs are then excised at the hip joint by blunt dissection and femurs and tibias separated by

cutting the knee joint. The paw is cut away from the tibia, the remaining skin is peeled off, and muscle and connective tissues are removed by sliding forceps down the bone while it is firmly held on a sterilized paper towel. The muscle and tissue remaining at the bottom of the bone are finally removed with scissors.

12. Alternatively, DCs can be isolated by mice spleens as reported in ref. [37].
13. The marrow flush should be repeated a couple more times, with additional complete medium or with the media in the marrow collection Petri dish, until the bone turns into a brighter and more translucent white.
14. BM-derived clumps retained in the strainer can be easily dispersed and recovered by using the rubber tip of the syringe plunger and rinsing the cell strainer with complete medium.
15. Health, age, and transplant status of mice may affect BM yields. Usually 2–6 × 10^7 BM cells can be collected from two tibias and two femurs.
16. The optimal culture period to generate BM-derived DCs with GM-CSF was reported to be about 7–10-days [38, 39].
17. As good practice, the washings and medium changes should be performed carefully; otherwise, the survival rate and number of the BM-derived DCs will be reduced.
18. Highly adherent cells (which are considered to be macrophages) should not be collected, and thus, if possible we recommend to not pipette too forcefully.
19. OT-1 mice express a transgenic TCR designed to specifically recognize OVA peptide residues 257–264 (SIINFEKL) in the context of H-2K^b, thus resulting in MHC-I-restricted, OVA-specific, CD8 OT-1 T cell activation.
20. The buffer is prepared according to manufacturer's instructions as a solution containing PBS, pH 7.2, 0.5% bovine serum albumin, and 2-mM EDTA.
21. Buffer and reagent volumes should be scaled up accordingly for higher cell numbers.
22. The sample volume is adjusted with additional separation buffer to achieve a minimum of 500 μL required for magnetic separation.
23. When experimentally required, the magnetically labeled non-CD8 T cell fraction can be flushed out by adding separation buffer and firmly pushing the plunger into the column.
24. At this step apoptosis induction should be confirmed by flow cytometry analysis. We suggest to perform an annexin V-PI apoptosis assay.

25. If possible, we recommend to not increase the co-culture surface and volume to promote cancer cell–DC interactions and ensure optimal phagocytic conditions.
26. Since physiologic phagocytosis occurs at 37 °C, at this step co-culture at 4 °C has been used as a negative control of uptake. Additionally, we also pulsed BM-derived DCs with 2 μg mL^{-1} of SIINFEKL peptide as a positive experimental control for DC maturation.
27. From this step onward, we recommend to work in the dark to preserve the fluorochrome signal and avoid photobleaching.
28. Optimal antibody concentration needs to be determined by titration.
29. Alternatively, other cell viability dyes (violet, aqua, green) not overlapping with used fluorescences could be employed.
30. According to the spectral properties of the vitality dye alternative lasers could be employed.
31. It is of extreme importance to avoid overlapping fluorescences as these can invalidate flow cytometry acquisition and analysis.

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Chapter 3

Retroviral Transduction of Human Primary T Cells Followed by Real-Time T-Cell-Mediated Cancer Cell Cytolysis Analysis

Anne Rahbech, Reno Debets, Per thor Straten, and Marlies J. W. Peeters

Abstract

Retroviral transduction is a highly useful tool to genetically engineer hard-to-transfect human primary cells. Here, we transduce human primary T cells with a tumor-specific T cell receptor. This creates a useful tool to analyze T cell–cancer cell interactions, such as cytolysis analysis using xCELLigence technology.

Key words Retrovirus, Transduction, T cells, xCELLigence, Cytolysis

1 Introduction

A retrovirus is characterized by its ability to retrotranscribe its RNA genome into cDNA, which can be stably integrated into the host cell. Retroviral transduction of human primary T cells is a widely used tool, which exploits the characteristics of retrovirus and provides a long-term manipulation of the host genome [1].

To make the retrovirus capable of replication, three major reading frames are needed: Gag, Pol, and Env [2, 3]. These can either be co-transfected together with the virus into the packaging cells or you can use a packaging cell line which already expresses the retroviral structure proteins. Here, we use the packaging cell line Platinum A, which stably produces a high yield of gag, pol, and env, creating a more simple and stable system.

Retroviral transduction is carried out over several steps. First, packaging cells are transfected with the virus to start the production of virus particles. Next, the newly produced virus is harvested from the packaging cells and transferred to the host cells. In this chapter, we use human primary T cells as target cells. To enhance the uptake of the virus, we use the calcium precipitation method. Prior to transduction, T cells have been stimulated with anti-CD3/CD28 beads, as efficient infection with the retrovirus and integration into the genome requires actively dividing host cells [4]. In this

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protocol, we transduce activated human primary T cells with a TCR specific for the melanoma antigen gp100 [5, 6].

After successful transduction, the killing capacity of the specific T cells can be tested [7]. The most used method for measuring cytotoxicity is the chromium release analysis, which was developed back in 1968 [8]. However, this method requires handling of radioactive material, and it is limited to a short time window of cytolytic analysis. Here, we use the Real-Time Cell Analysis xCELLigence system to follow the cytotoxicity of gp100TCR+ T cells against the gp100-expressing melanoma cancer cell line FM3 for 40 h.

2 Materials

Everything should be performed and prepared under sterile conditions. The transduction protocol should be carried out in a GMO Class 2 laboratory.

2.1 Culturing of Packaging Cells

1. Packaging cells: Platinum A cell line or other 293T-based packaging cell lines.
2. Culture medium for packaging cells: DMEM +10% fetal bovine serum (FBS), used for Platinum A cells.
3. Cell culture flasks or plates: tissue-culture treated.
4. Trypsin +0.25% ethylenediaminetetraacetic acid (EDTA).
5. Cell incubator: 37C°, 5% CO_2.

2.2 Transfection

1. Calcium Phosphate Transfection kit.
2. FACS tubes.
3. Vector including construct of interest.
4. Vortex.

2.3 Isolation and Activation of T Cells

1. Peripheral blood mononuclear cells (PBMCs).
2. Cell counter or microscope and counting plates.
3. Eppendorf tubes.
4. CD3/CD28 beads: DynaBeads® Human T-Activator CD3/CD28, Thermo Fisher Scientific.
5. Phosphate-buffered saline (PBS) + 5% human serum (HS).
6. Head-over-head tumbler: HulaMixer.
7. Magnet for Eppendorf tubes.
8. Culture plate: tissue-culture-treated plate.
9. Cell culture medium: X-vivo +5% HS + 100 U/mL IL-2 + 5 ng/mL IL-15.

2.4 Preparing Plates for Transfected Cells

1. Non-tissue-culture-treated (NTC) 24-well plate.
2. RetroNectin reagent: 10 μg/mL in PBS.
3. Parafilm.

2.5 Transduction of T Cells

1. Blocking buffer: PBS + 2% BSA (filtrated).
2. 0.45 μM filter.
3. Centrifuge: should be able to run at 1500 g force at 32 C° (slow acceleration (4) and low brake (3)).

2.6 Test Transduction Efficiency by Flow Cytometry

1. Antibody against the construct of interest.
2. Other relevant surface antibodies.
3. FACS buffer: PBS + 2% FBS.

2.7 xCELLigence Assay

1. xCELLigence RTCA SP instrument.
2. RTCA software: The "Pro" version is needed for measuring cytolysis.
3. RTCA E-plates.
4. Target cells (in this study: FM3 cancer cells): preferable adherent cells.
5. Effector cells (in this study: gp100TCR engineered T cells).
6. Warming plate.
7. Culture medium for your target cells: RPMI+10% FBS is used for FM3 cancer cells.

3 Methods

3.1 Seeding of Packaging Cells: Day 1

1. Harvest and count the packaging cells (Platinum A).
2. Plate cells at different concentrations in several T75 flasks, such as 1×10^6, 2×10^6, and 3×10^6 cells per flask in 11 mL of cell culturing media (DMEM+10% FBS) (*see* **Note 1**).
3. Incubate at 37C°, 5% CO_2.

3.2 Transfection of Packaging Cells: Day 2

1. Carefully look at the cells and inspect confluency. If they do not look apoptotic and are adhered, change the medium with some fresh prewarmed DMEM (*see* **Note 2**).
2. Thaw the kit for Calcium Phosphate Transfection method.
3. Prepare 2 FACS tubes (1 for DNA and 1 for HEPES).
4. Needed amounts: DNA, 20 μg; $CaCl_2$, 62 μL; dH_2O, top up to 500 μL in total.
5. First add the DNA and water to the tube, mix well, and then add the $CaCl_2$ and mix again by pipetting.

6. Add 500 μL of HBS buffer to the second FACS tube.
7. Gently vortex the tubes containing the HBS buffer. Continue while slowly adding the prepared DNS solution dropwise to the HBS tube. When DNA addition is complete, the solution should appear slightly opaque.
8. Incubate the solution at room temperature for 30 min.
9. Pick a flask of Platinum A cells with a confluency of around 60%.
10. Vortex the DNA-HBS solution and immediately after; add it (1 mL) dropwise to the cells in the flask. Gently swirl the flask to ensure even distribution.
11. Incubate 37C°, 5% CO_2 until the next day.

3.3 Isolation and Activation of T Cells: Day 2

1. Thaw 40 × 10^6 PBMCs and resuspend in PBS + 5% human serum (H.S.).
2. Count cells and determine amount of CD3+ cells. Assume around 50% are CD3+.
3. Adjust cell number to 10 × 10^6 cells/mL in an Eppendorf tube.
4. Prepare CD3/CD28 beads.
5. Calculate how many CD3/CD28 beads should be added. CD3/CD28 bead stock concentration is 40.000 beads/μL. For isolation of CD3+ cells, a bead to cell ratio of 2:1 is needed. For example, for 10 × 10^6 cells add 250 μL beads.
6. Wash the appropriate amounts of beads using PBS + 5% H.S. and resuspend them in the original volume in PBS + 5% H.S.
7. Add the beads to the PBMCs.
8. Incubate the cells with the beads on a head-over-head tumbler (HulaMixer) (10 RPM, 44° and 4°) for 30 min at room temperature (RT).
9. Place cells on a suitable magnet to remove non-bead-bound cells (CD3-cells).
10. Count cells and adjust the concentration to 1 × 10^6 cells/mL of X-VIVO +5% H.S + 100 U/mL IL-2 + 5 ng/mL IL-15.
11. Plate the cells in a tissue-culture-treated (TCT) 24-well plate (*see* **Note 3**).

3.4 Coat Plates with RetroNectin: Day 3

1. Thaw RetroNectin (10 μg/mL in PBS).
2. Add 400 μL (4 μg) RetroNectin per well of a non-tissue-culture-treated (NTC) 24-well plate.
3. Wrap the plate with parafilm and incubate it overnight at 4 °C.

3.5 Give New Media to Packaging Cells: Day 3

1. Warm up medium (DMEM+10% FBS) to 37 °C.
2. Very carefully take out the flask of transfected packaging cells from the incubator, keeping the bottle horizontal (*see* **Note 2**).
3. Very carefully remove around 7 mL of medium from the flask and add 7 mL of prewarmed medium.

3.6 Transduction of Human Primary T Cells (Hit 1): Day 4

1. Prewarm medium (DMEM +10% FBS) to 37 °C.
2. Transfer RetroNectin from the coated plate to a new NTC 24-well plate. Wrap it with parafilm and incubate at 4 °C overnight. This is for the second hit of transduction.
3. Block the wells with 500 μL/well of PBS + 2% BSA (filtrated with 0.45 μm filter) for 30 min at RT.
4. Harvest virus supernatant (11 mL) from the packaging cells and carefully add new prewarmed DMEM+10% FBS (11 mL) to the flask. Return the cells to the 37 °C incubator.
5. Filtrate virus supernatant using a 0.45-μM filter.
6. Remove blocking buffer from the NTC plate and add 1 mL of PBS. Remove this again right after (*see* **Note 4**).
7. Add 2 mL of virus supernatant per well of the RetroNectin-coated plate (*see* **Note 5**). Add 2 mL of DMEM+10% FBS to the wells for mock transduced cells.
8. Wrap the plate with parafilm and centrifuge the plate for 1 h at 3000 rpm (1500 g) at 32 °C (slow acceleration (4) and low brake (3)).
9. Harvest T cells and count.
10. Spin cells down and adjust the concentration to 0.5×10^6 cells/mL of X-VIVO +5% H.S + 100 U/mL IL-2 + 5 ng/mL IL-15.
11. Remove 1.5 mL of virus supernatant on the coated 24-well plate.
12. Add 1 mL per well of T cells (0.5×10^6 cells).
13. Seal the plate with parafilm and spin for 1 h at 2500 rpm (1000 g), 32 °C (slow acceleration (4) and low brake (3)).
14. Remove the parafilm and incubate cells overnight at 37 °C, 5% CO_2.

3.7 Transduction of Human Primary T Cells (Hit 2): Day 5

1. Repeat **step 2–8 from section 3.6**. Discard the packaging cells and freeze down the RetroNectin (*see* **Note 6**).
2. After centrifugation, remove 1.5 mL of the virus supernatant from the plate.
3. Resuspend the transduced T cells from hit 1 and transfer them to the new RetroNectin-coated plate.

4. Seal the plate with parafilm and spin for 1 h at 2500 rpm, 32 °C (slow acceleration (4) and low brake (3)).
5. Incubate overnight at 37 °C, 5% CO_2 (*see* **Note 7**).

3.8 Transfer Cells to TCT Plate

1. Transfer cells to a TCT 24-well plate.
2. Look at the cells under a microscope. If they look fine and are growing, split them 1:1. This is done by resuspending cells and transferring half of the volume from the well to a new well. The volume is replaced with new media containing double concentrations of cytokines.
3. Incubate at 37 °C, 5% CO_2 (*see* **Note 8**).

3.9 Test Transduction Efficiency by Flow Cytometry: Day 8

1. Resuspend cells, count them, and transfer around 5×10^5 cells to a FACS tube.
2. Wash cells twice in FACS buffer.
3. Stain cells for surface markers including the marker of the construct, e.g., NiR, CD3, and Vβ14.
4. Incubate dark for 30 min at 4 °C.
5. Wash cells twice with FACS buffer and resuspend in 100 μL of FACS buffer.
6. Acquire on a flow cytometer (*see* Fig. 1).

3.10 Sort Cells

1. To get a purer culture of successfully transduced cells, it is recommended to sort the cells (*see* Fig. 1c). This can be done in several ways, such as sorting by magnetic beads or by flow cytometry.

3.11 Culturing of Cells

1. Inspect the cells and evaluate the color of the medium and notice if they are forming clusters. If that is the case, split them 1:1. This is done by resuspending cells and transferring half of the volume from the well to a new well. The volume is replaced with new media containing double concentrations of cytokines.

3.12 Titration of Target Cells in xCELLigence RTCA SP System (See Note 9)

1. Prewarm cell culture media to 37 °C.
2. Add 100 μL of cell culture media to each well of an E-plate.
3. Place the plate in the xCELLigence RTCA SP instrument (SP system) while preparing the cells, but do not run the background check yet.
4. Harvest and count target cells.
5. Take the needed number of cells into a sterile Eppendorf tube and adjust volume to 800 μL.

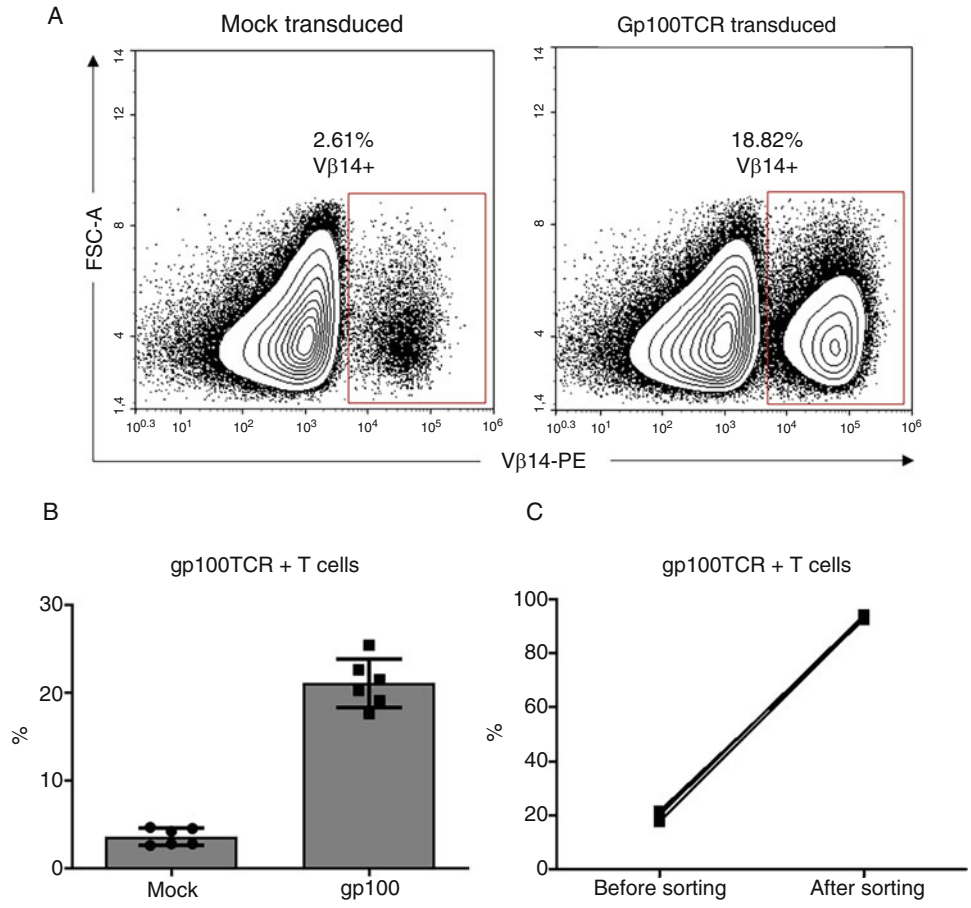

Fig. 1 Transduction efficiency shown by surface expression of Vβ14 as a marker of gp100TCR+ cells. Mock cells are used as a negative control. (**a**), representative plot of mock and gp100TCR transduced T cells. (**b**), transduction efficiency from six independent experiments. (**c**), surface expression of Vβ14 (gp100TCR+ cells) before and after sorting using MACS anti-PE beads, $n = 3$

6. Prepare Eppendorf tubes for the titration by adding 400 μL of media to the number of tubes corresponding to the number of titration steps you want (*see* **Note 10**).
7. Make serial dilution by resuspending and transferring 400 μL of cells from the first tube into the next. Repeat this until you have the needed number of dilutions. Discard 400 μL of cells from the last tube.
8. Run background check. If passed, take out the plate.
9. Add 100 μL of cells from each Eppendorf tube to the appropriate wells in the E-plate. Plate each condition/cell number in triplicates.

10. Let cells rest in the bench for 30 min before adding the E-plate to the SP system.
11. Run experiment for around 3 days.

3.13 Cytolysis Assay in xCELLigence

1. Choose the appropriate number of target cells according to the titration (*see* **Note 9**).
2. Prewarm cell culture media to 37 °C.
3. Add 100 μL of cell culture media to each well of an E-plate.
4. Place the plate in the SP system while preparing the cells, but do not run the background check yet.
5. Harvest and count target cells.
6. Take the needed number of cells into a sterile tube and adjust volume. One hundred microliter will be used for each well.
7. Prepare the setup in the xCELLigence RTCA software (*see* Table 1).
8. Run background check.
9. Add 100 μL of target cells per well according to your experimental setup (*see* **Note 11**).
10. Let cells rest in the bench for 30 min before adding the E-plate to the SP system.
11. Start the experiment and run it until the target cells have attached and initiated proliferation (*see* **Note 10**).
12. Prewarm cell culture medium.
13. Harvest and count the effector cells.
14. Calculate the appropriate number of cells needed for the specific experimental setup.
15. Make a serial dilution of the cells in a 96 well plate (*see* **Notes 12** and **13**).
16. Prepare a warming plate to 42 °C.

Table 1
Example of a setup in the xCELLigence RTCA software

Step	# sweeps	Interval	Time	Total time
1: Background check	1	1 s	–	00:00:01
2: Target cell adhesion	157	10 min	26 h	26:00:01
3: Addition of effector cells	97	5 min	8 h	34:00:01
4: Killing	161	15 min	40 h	74:00:01
5: Killing	49	30 min	24 h	98:00:01

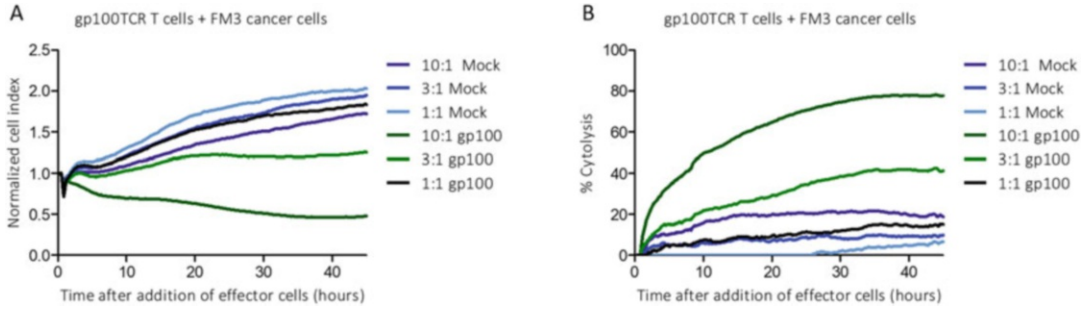

Fig. 2 Real-time in vitro cytolysis of FM3 cancer cells after the addition of gp100TCR transduced T cells. Shown with different effector to target ratios (E:T = 10:1, 3:1, and 1:1). The values are normalized to the growth of FM3 cells alone. (**a**), normalized cell index. (**b**), cytolysis of FM3 target cells by the gp100TCR effector T cells. Values are normalized to the timepoint right before addition of effector cells

17. Pause the experiment and take out the E-plate from the SP system and work fast from now on.
18. Remove 100 μL of medium from all wells.
19. Add 100 μL of prewarmed cell medium to the wells without effector cells (only target cells, negative control).
20. Add 100 μL of Triton X (10%) to wells for full cytolysis (positive control).
21. Transfer the right cells from the prepared 96-well plate to the respective wells in the E-plate.
22. Put the E-plate back into the SP system and resume the readout for the desired time (*see* **Note 14**).
23. When the experiment has ended, analyze the data in the SP software or export it to analyze it elsewhere (*see* **Note 15** and Fig. 2).

4 Notes

Transduction protocol

1. Packaging cells should be around 60% confluent at day 2. If they are fully confluent or less confluent, it can affect the transfection/virus production.
2. The transfection might be hard to the packaging cells, and they should therefore be handled with extra care. It is therefore recommended to prewarm cell media and to keep the flask horizontal when possible during all steps.
3. It is a good idea to plate some extra cells than the amount you need for transduction at day 4.

4. Do not allow the RetroNectin-coated wells to dry out. Remove PBS only when you can add virus supernatant right away.
5. Virus supernatant can also be snap-frozen (liquid nitrogen) and stored at −80 °C for later use. For bulk production, do one test transduction of virus supernatant batch before use.
6. RetroNectin can be thawed and refrozen three times. Throw it away after you thawed it and used it for the third time.
7. If needed, the incubation could be decreased to 6 h, and the following step could be carried out afterwards.
8. Cells should be cultured/split every other day (Monday, Wednesday, and Friday). They can be in culture for up to 3 weeks after transduction. For important experiments, use them as soon as possible after the transduction. Preferably within the first week.

   xCELLigence

9. It is important to make a cell titration of the target cells as the first thing to find the suitable number of cells to add per well. Cell number should not be too high, as they will overgrow and start dying, and it should not be too low, as we want a cell index of around 1 the following day, when adding the target cells.
10. Add effector cells when the target cells have reached a cell index around 1, but before they reach the plateau (*see* Fig. 3). The time it takes for the target cells to reach this level is dependent on the cell line and should be optimized.
11. For the control wells, such as the "full lysis" and "background," the highest number of effector cells should be used.
12. An example of a serial dilution of effector cells could be an effector/target ratio (E:T) of 10:1, 3,3:1, and 1:1.

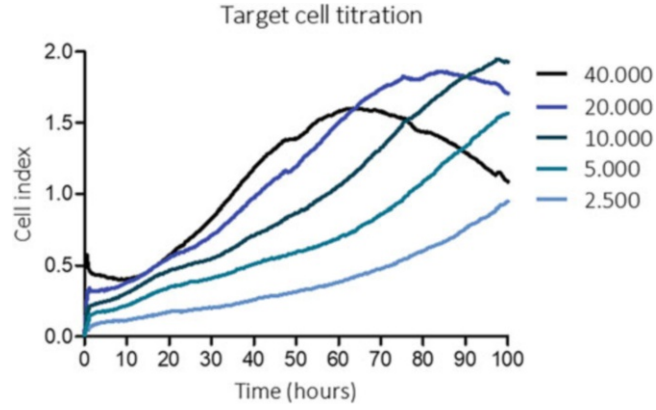

Fig. 3 Example of a titration of target cells in xCELLigence shown with the melanoma cell line FM3

13. It is recommended to prepare the effector cells in a normal 96-well cell culture plate, as this will minimize the time where the E-plate is out of the incubator and thereby keeps a more consistent temperature.
14. At some point the effector cells and the target cells in the plate will start to die due to overgrowth and too little space in the wells. The experiment should be stopped before this as this will lead to unreliable data.
15. Normalize the data to the last timepoint before effector cells or treatment was added.

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Chapter 4

Expansion and Retroviral Transduction of Primary Murine T Cells for CAR T-Cell Therapy

Pauline Loos, Lauralie Short, Gillian Savage, and Laura Evgin

Abstract

The development of chimeric antigen receptor (CAR) T cells has been a revolutionary technology for the treatment of relapsed and refractory leukemias and lymphomas. The synthetic CAR molecule redirects T cell function toward tumor surface-expressed antigens through a single-chain variable fragment (scFv) fused to CD3z and intracellular costimulatory domains. Here, we describe a protocol for the generation of CAR T cells using primary mouse T cells and a gammaretroviral vector encoding a CAR transgene. This protocol outlines several transduction and expansion methods based on the use of two transduction enhancers, RetroNectin® and Vectofusin®-1, and cell culture systems such as conventional plates or G-Rex® devices.

Key words Chimeric antigen receptor (CAR), T cell, Transfection, Transduction, Gamma-retroviral vector

1 Introduction

CAR T-cell therapy capitalizes on synthetic biology to enable T cells to recognize and eradicate cancer cells. This adoptive immunotherapy has reshaped the treatment of relapsed or refractory B-cell malignancies, but leveraging the full potential of CAR T cell therapy remains hampered by limitations in our current understanding of the underlying biological determinants of efficacy. Immune-competent mouse models of cancer allow for the characterization of CAR T cells from multiple tissues, across time points, in an immune-relevant context, and provide complementary information to that obtained from patient samples and in vitro and xenograft studies with human T cells. Herein, we focus on the generation of murine CAR T cells that can be used in widely available mouse models.

Although gammaretroviruses and lentiviruses are both used for human T cell transduction preclinically and clinically, retroviruses

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are the preferred modality for primary mouse T cells as they yield more efficient transduction [1–3]. Herein, we describe a transduction protocol based on the use of the MSGV1 gammaretrovirus transfer plasmid [4] and the pCL Eco packaging plasmid to provide the accessory gag, pol, and ecotropic envelope genes in trans [5], the latter of which is readily available through Addgene. We have used this protocol to generate CAR T cells with multiple specificities, including EGFRvIII and CD19, and have used the Thy1.1 marker expressed from an IRES to identify transduced T cells. These methods have enabled us to understand how to combine cell therapies with other types of immunotherapies that engage multiple innate and adaptive immune components, such as oncolytic viruses [6, 7].

Modification of primary cells with retroviral vectors often requires the presence of transduction-enhancing reagents. Here, we describe protocols based on the use of two different enhancers, RetroNectin® (Clontech) and Vectofusin®-1 (Miltenyi). RetroNectin® (also described as CH-296) is a recombinant fragment of human fibronectin with three functional domains: the RGDS motif-containing cell adhesion domain, the heparin-binding (H) domain, and the CS-1 cell adhesion domain [8]. The molecule acts as a bridge as virus particles binding the H-domain are brought into close proximity with cells interacting with the RGDS or CS-1 domains through VLA-5 and VLA-4 on target cells, respectively. Vectofusin®-1 is a synthetic cationic amphipathic peptide that induces aggregation of viral particles and facilitates the adhesion and fusion of viral and cellular membranes [9–11]. While RetroNectin® must be precoated onto cell culture surfaces to enhance transduction via spinoculation, Vectofusin®-1 is a soluble reagent that can enhance transduction under static conditions.

T cells can be efficiently spinoculated and expanded in 24-well plates; however, static transduction enhancers enable the use of G-Rex® devices to grow T cells and avoid splitting the cells during the expansion step. The G-Rex® has a silicone membrane at the base of the vessel and facilitates efficient gas exchange in a manner that is independent of the volume of media above the cells [12]. As such, the cells can grow to a greater density without media exchanges, and these vessels have been ubiquitously used to expand preclinical and GMP-grade materials, including T cells [13–17].

In this chapter, we have included methods for CAR T cell generation using a gammaretroviral vector that make use of different cell expansion and transduction enhancer products. We describe transduction by spinoculation using the transduction enhancer RetroNectin® in **Method A** and Vectofusin®-1 in **Method B**. Finally, in **Method C** we describe static transduction with the transduction enhancer Vectofusin®-1 using regular 24-well plates (**C1**) or the G-Rex® devices (**C2**). Each method has advantages and disadvantages which are outlined in Table 1.

Table 1
Advantages and disadvantages of various transduction and cell expansion processes

	Processes/ format	Advantages	Disadvantages
Transduction method	Spinoculation	Yields the highest transduction rate when associated with transduction enhancers	Time consuming May induce cell stress
	Static	Time-saving	Lower transduction rate than spinoculation
Transduction enhancer	RetroNectin®	Yields very high transduction rates	Pre-coating the plate is time consuming Spinoculation may induce cell stress
	Vectofusin®-1	High-efficiency transduction with spinoculation Moderate transduction efficiency with static conditions	Requires serum-free media for the transduction step
Expansion format	24-well	Easy to source, low cost, amenable to small or large batch production	Scaling many 24-well plates can be cumbersome
	G-Rex®	No media changes during the process. Provides gas exchange to the cells to maximize expansion	Cannot visualize the cells under the microscope Cannot be spinoculated to enhance transduction Mouse T-cell culture is shorter than human T cell culture and may not maximize the long term advantage of the G-Rex®

2 Materials

2.1 293T Cell Culture

1. DMEM supplemented with 10% FBS (v/v), store at 4 °C.
2. 1× PBS: without Ca/Mg; store at room temperature.
3. 0.25% trypsin–EDTA; store at 4 °C.
4. Poly-D-lysine-coated plates (*see* **Note 1**); store at room temperature.
5. 50-mL tubes.

2.2 Transfection

1. DMEM supplemented with 10% FBS (v/v); store at 4 °C.
2. Lipofectamine® 2000 transfection agent; store at 4 °C.
3. Opti-MEM®; store at 4 °C.
4. MSGV1 1D3-28BBz IRES Thy1.1: retroviral transfer plasmid encoding the CAR cassette and the marker gene (anti-mouse CD19 CAR with CD3); store at −20 °C.
5. pCL Eco: retroviral packaging plasmid; store at −20 °C.

6. CAR T-cell media:
   (a) RPMI-based CAR T cell media (**Method A**).
      (i) RPMI 1640 with L-glutamine supplemented with:
         1. 100 U/mL penicillin/streptomycin.
         2. 1× MEM nonessential amino acid solution.
         3. 1 nM sodium pyruvate.
         4. 50 μM BME.
         5. 10% (v/v) FBS.
   (b) Serum-free media (**Methods B and C**) (*see* **Note 2**):
      (i) TexMACS™ media supplemented with:
         1. 100 U/mL penicillin/streptomycin
7. 50-mL tubes.

2.3 Splenocyte Collection

1. Mouse spleen (i.e., from C57BL/6 or Balb/c background) freshly collected (*see* **Note 3**).
2. RPMI; store at 4 °C.
3. ACK (ammonium–chloride–potassium) lysis buffer; store at room temperature.
4. 100-μM cell strainer.
5. 10-mL syringe.
6. 50-mL tubes.

2.4 T-Cell Activation and Culture

1. RPMI-based CAR T-cell media (*see* Subheading 2.2).
2. Concanavalin A (ConA) (2.5 mg/mL stock); store at −20 °C.
3. Murine recombinant cytokines: IL-7 (50 μg/mL stock) and IL-15 (50 μg/mL stock); store at −20 °C (*see* **Note 4**).
4. Tissue-culture-treated 24-well plates.
5. 50-mL tubes.

2.5 Transduction

Method A: Spinoculation Using Transduction Enhancer RetroNectin®

1. RetroNectin® (1 mg/mL stock); store at −20 °C.
2. Non-tissue-culture-treated 24-well plates.
3. 1× PBS: without Ca/Mg; store at room temperature.
4. Parafilm.
5. High-speed swinging-bucket centrifuge, with adapters for 24-well plates.
6. PBS supplemented with 2% bovine serum albumin (BSA) (w/v) and filter sterilized.
7. 50-mL tubes.

Method B: Spinoculation Using Transduction Enhancer Vectofusin®-1

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. Tissue-culture-treated 24-well plates.
3. 1× PBS: without Ca/Mg; store at room temperature.
4. Parafilm.
5. High-speed swing-bucket centrifuge, with plate adapters.
6. 50-mL tubes.

Method C: Static Using Transduction Enhancer Vectofusin®-1

C1 with regular tissue culture 24-well plates

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. Tissue-culture-treated 24-well plates.
3. 50 mL tubes.

C2 with G-Rex® tissue culture 6-wells

1. Vectofusin®-1 (1 mg/mL stock); store at –80 °C.
2. G-Rex® 6 multi-well cell culture (*see* **Note 5**).
3. 50-mL tubes.

2.5.1 Validation of Transduction

1. Fluorescently labelled anti-mouse antibodies against Thy1.1, CD4, CD8, CD44, CD62L, and a fixable live/dead staining dye (*see* Table 2 for a sample panel).
2. FACS buffer (1% BSA [w/v], 2 mM ethylenediaminetetraacetic acid [EDTA], 0.05% NaN_3 [w/v]).
3. 50-mL tubes.
4. 5-mL round-bottom polystyrene tubes with caps.

Table 2
Example antibody panel with associated fluorochromes and clones used to evaluate the CAR expression on T cells by flow cytometry

Antigen	Clone	Conjugate	Source	Dilution
Fixable live/dead stain		NIR	Biolegend	1/1500
CD4	GK1.5	BV421	Biolegend	1/750
CD8β	53–5.8	PerCP-Cy5.5	Biolegend	1/500
Thy1.1	OX-7	PE	Biolegend	1/1000
CD44	IM7	BV786	Biolegend	1/500
CD62L	MEL-14	BV605	Biolegend	1/500

5. 96-well round-bottom plate.
6. High-speed centrifuge with a swinging-bucket rotor, with plate adapters.
7. Flow cytometer.

3 Methods

3.1 Retrovirus Production

The following protocol is for transfecting one 10-cm plate, which yields around 9–12 mL of viral supernatant. Scale as required. Thaw fresh 293T cells approximately 1 week prior to the date they are needed.

3.1.1 Day -1: Plate 293 T Cells for Transfection

1. Collect 293T cells: Aspirate the media and wash the cells with PBS. Use 0.25% trypsin–EDTA to detach the cells from the vessel. Neutralize the trypsin with DMEM supplemented with 10% FBS and resuspend the cells. Transfer the cells to a 50-mL tube.
2. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend the cells in 10 mL of DMEM supplemented with 10% FBS. Count the cells using a hemocytometer.
3. Dispense 3.5×10^6 cells per poly-D-lysine-coated 10-cm plate in approximately 10 mL of DMEM supplemented with 10% FBS. Rock the plate(s) in different directions to evenly distribute the cells and incubate at 37 °C overnight.

3.1.2 Day 0: 293T Cell Transfection

1. Aspirate the media on the 293T cells and add 10 mL of fresh DMEM supplemented with 10% FBS within 30 min of transfection.
2. Prepare two 50-mL tubes with 1.5 mL of Opti-MEM® in each (adjust for the number of plates as required):
   - Tube #1: Add 60 μL of Lipofectamine.
   - Tube #2: Add 14.1 μg of MSGV1 1D3-28BBz IRES Thy1.1 + 9.9 μg of pCL Eco.

   Incubate for 5 min at room temperature.
3. Add the contents of Tube#2 to Tube#1. Mix only very gently. Incubate for 20 min at room temperature.
4. Add 3 mL of the lipid/DNA complex dropwise to the 293T cells. Incubate the plate(s) at 37 °C overnight (*see* **Note 6**).

3.1.3 Day 0: Splenocyte Collection

1. Mash the spleen through a 100-μM filter with the syringe plunger onto a 50-mL tube and wash twice with 5 mL of RPMI.

2. Spin at 300×*g* for 5 min at room temperature. Resuspend in 1 mL of ACK buffer. Incubate for 1 min at room temperature and then add 9 mL of RPMI.
3. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend the cells in 20 mL of RPMI. Count the cells using the hemocytometer.
4. Spin at 300×*g* for 5 min at room temperature. Aspirate the supernatant and resuspend at 2×10^6 cells/mL in RPMI-based CAR T-cell media + ConA (2.5 μg/mL) + murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
5. Plate 2 mL per well (total 4×10^6 per well) and put the plate (s) into the 37 °C incubator overnight.

3.1.4 Day 1: Replace Media on 293T Cells

1. Aspirate the media from the transfected plate(s) (*see* **step 4**, Subheading 3.1.2).
2. Add 9–12 mL of RPMI-based CAR T-cell media (**Method A**) or TexMACS™-based CAR T-cell media (**Methods B** and **C**) (*see* **Note 7**) on the 293T cell plate along the vessel wall. Be careful not to disturb and detach the cells. Repeat for all other plates (*see* **Note 8**).
3. Incubate the plate(s) at 37 °C overnight.

3.2 Transduction

3.2.1 Method A: Spinoculation Using Transduction Enhancer RetroNectin®

Day 1: RetroNectin® Coating

1. Plate 400 μL of PBS with 25 μg/mL of RetroNectin® in each well of a non-tissue-culture-treated 24-well plate.
2. Put the plate at 4 °C overnight.

Day 2: Harvest Retrovirus Supernatant

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the cell pellet.

Day 2: T-Cell Transduction

1. Remove the PBS/RetroNectin® solution from the 24-well plate by aspirating. Add 0.5 mL of PBS supplemented with 2% BSA per well and incubate for 10–30 min at room temperature.
2. Remove PBS/BSA solution and wash each well with 1–2 mL of PBS.
3. Harvest T cells by gentle pipetting and pool them in a 50-mL tube. Count the cells using a hemocytometer. Set aside some cells to use as an untransduced control.

4. Spin the desired number of T cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the cells in viral supernatant at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) (*see* **Note 7**).
5. Aspirate the PBS from the 24-well plate and transfer 1 mL of T cells in retrovirus supernatant per well.
6. Use parafilm to seal the plate. Spin the plate(s) for 90 min at 800×*g*; acceleration 4, brake 0; at 32 °C using biohazard rotor covers.
7. After the spin, add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to each well.

Day 3: Expand T Cells

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new tissue-culture-treated 24-well plate. Add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.2.2 Method B: Spinoculation Using Transduction Enhancer Vectofusin®-1

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the pellet.

Day 2: Harvest Retrovirus Supernatant

Day 2: T-Cell Transduction

1. Harvest T cells by pipetting and pool them in a 50-mL tube. Count the cells using a hemocytometer. Set aside some cells to use as an untransduced control. Spin the desired number of cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the T cells in PBS to remove any residual serum, and spin again at 300×*g* for 5 min at room temperature.
2. Add 10 μg/mL of Vectofusin®-1 in viral supernatant and incubate for up to 5 min at room temperature. Resuspend the washed T cells in viral supernatant/Vectofusin®-1 at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
3. Transfer 1 mL per well in a tissue-culture-treated 24-well plate.
4. Use parafilm to seal the plate. Spin for 90 min at 800×*g*; acceleration 4, brake 0; at 32 °C using biohazard rotor covers.
5. After the spin, add an additional 1 mL of RPMI-based media (with FBS) with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to each well in the 24-well plate.

Day 3: Expand T Cells

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new 24-well plate. Add 1 mL of RPMI-based CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.2.3 Method C: Static Transduction Using Vectofusin®-1

1. Collect the retrovirus supernatant with a pipette in a 50-mL tube. Spin at 500×*g* for 10 min at room temperature.
2. Transfer the supernatant to a new 50-mL tube and leave at least 1 mL behind so as to not disturb the pellet.

Day 2: Harvest Retrovirus Supernatant

Day 2: T-Cell Transduction

1. Harvest the T cells by pipetting gently and pool them in a 50-mL tube. Count the cells using a hemocytometer. Spin the desired number of cells to be transduced at 300×*g* for 5 min at room temperature. Resuspend the T cells in PBS to remove any residual serum, and spin again at 300×*g* for 5 min at room temperature. In parallel, set aside some T cells to use as an untransduced control.
2. Add 10 μg/mL of Vectofusin®-1 to the retrovirus supernatant and incubate for 5 min at room temperature. Resuspend the washed T cells in the retrovirus supernatant at 1×10^6 cells/mL and add murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL).
3. Aliquot 1 mL (1×10^6 cells) per well of a tissue culture 24-well plate (**Method C1**) or 5×10^6 to 1×10^7 cells in at least a volume of 5–10 mL in each well of a G-Rex® 6-well cell culture plates (**Method C2**) (*see* **Note 5**).
4. Incubate for 4 h at 37 °C. Add an additional 1 mL of CAR media (with FBS) with cytokines to the 24-well plate (**Method C1**) and an additional 10–15 mL to each G-Rex® well (**Method C2**).

Day 3: Maintain T-Cell Activation (Only for **Method C1**)

1. Split cells by gently resuspending and pipetting half the volume of each well (1 mL of cells) into a new 24-well tissue-culture-treated plate. Add 1 mL of CAR media with murine recombinant cytokines IL-7 (10 ng/mL) and IL-15 (5 ng/mL) to all wells.

3.3 Evaluation of CAR Transduction

3.3.1 Day 4 or 5: Harvest CAR T Cells

1. Gently resuspend cells by pipetting. Collect all the cells in 50-mL tubes and count using a hemocytometer. Prepare aliquots of 1×10^6 cells for flow staining and transfer to a 96-well round-bottom plate.
2. Spin the plate at 800×*g* for 2 min and resuspend cells in 100 μL of a fixable live dead stain diluted in PBS. Incubate for 10–20 min at room temperature.

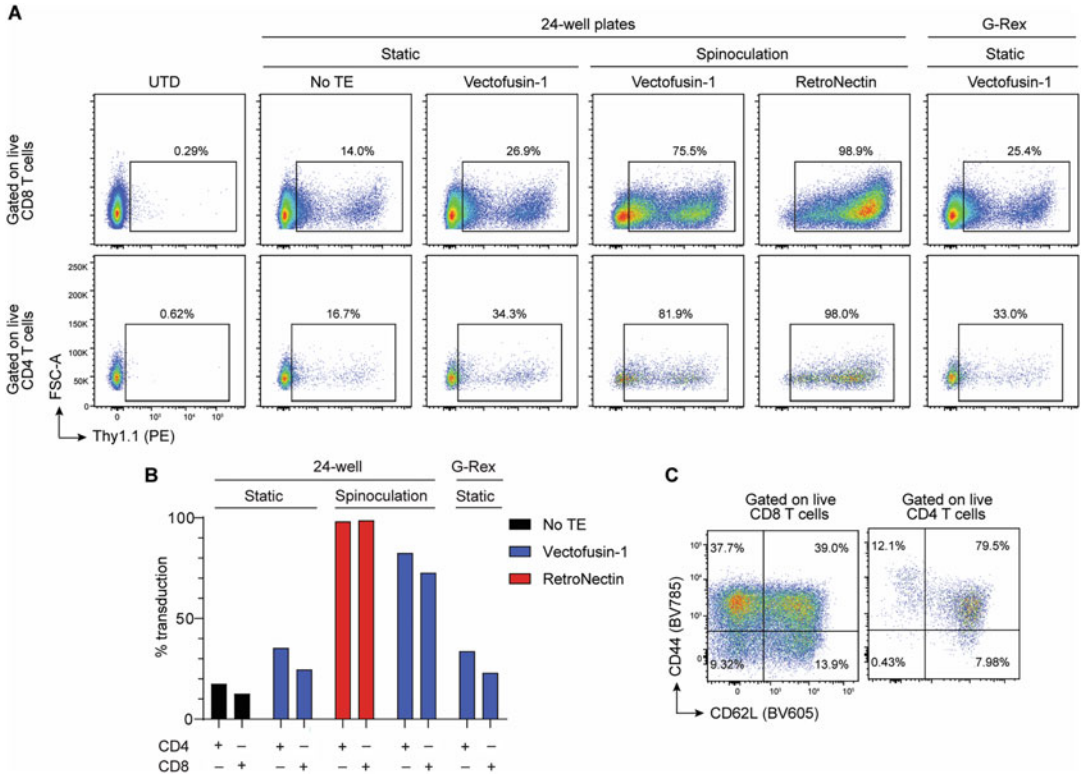

Fig. 1 On day 1, 9 mL of media was used to replace the transfection media. T cells were transduced in the presence of the indicated transduction enhancers and expanded in the indicated culture systems. Samples were run on a BD FACSymphony™ and analyzed using FlowJo 10 software. (**a**) Representative flow plots gated on live CD8 or CD4 T cells showing the expression of the CAR marker transgene Thy1.1 on day 4. (**b**) Transduction rates in CD4 and CD8 T cells from indicated transduction conditions. (**c**) Representative flow plots of the expression of CD44 and CD62L in CD8 and CD4 T cells following transduction by spinoculation in the presence of RetroNectin® (**Method A**). *TE* transduction enhancer; *UTD* untransduced

3. Spin the plate at 800×*g* and resuspend the cells in 100 µL of the antibody mastermix diluted in FACS buffer. *See* Table 2 for a sample antibody panel. Incubate for 20 min at 4 °C.
4. Spin the plate 800×*g* and resuspend cells in 150 µL of FACS buffer to wash the cells.
5. Spin the plate 800×*g* and transfer the cells to the 5-mL round-bottom tubes. Bring the volume up to ~400 µL and run on an appropriate flow cytometer. Refer to Fig. 1 and **Notes 9–11** for expected results.

4 Notes

1. Poly-D-lysine promotes 293T cell attachment and thus increases viral yield from the producer cells. Either pre-coated plates may be used or a poly-D-lysine solution may be used to directly coat the plates following the manufacturer's instructions.
2. The efficacy of Vectofusin®-1 is affected by the presence of serum in the media. Serum-free culture media such as TexMACS™ should be used to replace the transfection media on the 293 T cells on day 1 instead of RPMI-based media containing FBS.
3. While human T cells can be readily frozen and thawed, mouse T cells are less resilient to freezing and we have always used fresh T cells to maximize their viability and functionality.
4. Various cytokines can be used to expand the T cells. We have successfully used human IL-2 alone as well as combinations of mouse IL-7 (10 ng/mL) and mouse IL-15 (5 ng/mL) with or without mouse IL-21 (30 ng/mL) on day 0 only. IL-7, IL-15, and IL-21 are well documented to limit terminal differentiation of T cells and, in our hands, yield a better viability compared to human IL-2.
5. G-Rex® devices cannot be spun due to the silicone membrane at the bottom of the vessel.
6. From this point on, this supernatant has infectious retrovirus in it. All materials that come in contact with the retrovirus should be disinfected in 10% bleach.
7. The volume of media used to replace the transfection mix on day 1 can be adjusted to alter the viral titer. For example, for retroviruses with a small gene insert, 12 mL can be used, and the volume can even be diluted further (2/3 retrovirus supernatant +1/3 media). For instances where the titer needs to be maximized (i.e., the transgene size approaches the maximum retrovirus coding capacity, or when using Vectofusin®-1), the volume can be reduced to enhance the transduction efficiency.
8. In contrast to lentivirus which can readily be made as a large batch and stored at −80°C, the ecotropic retrovirus cannot be frozen without a significant loss of titer. Therefore, we prepare fresh retrovirus supernatant for each transduction.
9. As the T-cell expansion and transduction are extremely efficient, the yield on day 4 is >90% T cells and a sorting step is not typically performed. If desired, a magnetic bead-based sort for Thy1.1 or untouched T cells can be performed.

10. If 9 mL of media is used to replace the media on the 293T cells on day 1, the following transduction rates can be obtained:
    - **Method A:** >90% transduction
    - **Method B:** 70–85% transduction
    - **Method C:** 20–35% transduction
11. To illustrate a typical expansion and yield for **Method A**, the following example is provided:

    To treat three groups of six mice with 10^7 CAR T cells → 18 doses = 1.8×10^8 cells + excess for injection.

    Day 0: Harvest spleens from four donor mice.

    Transfect 8 × 10 cm plates of 293 T cells.

    Day 1: Coat 5 × 24-well plates with RetroNectin®.

    Day 2: Harvest T cells and expect to collect 70–90% of the total number of cells plated on day 0. Bring the retrovirus supernatant volume up to 120 mL. Spinoculate 5 × 24-well plates.

    Day 3: Split cells ½ into 10 × 24-well plates.

    Day 4: Harvest T cells and expect to collect approximately three times more cells than plated on day 2. Overall expected yield is ~4×10^8.

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Chapter 5

In Situ Decellularization of Tissues Applied to the Topographical Analysis of Tumor-Associated Extracellular Matrix

Alejandro E. Mayorca-Guiliani

Abstract

The extracellular matrix (ECM) is a network woven out of more than 1300 different proteins, of which ≈300 are structural. Their presence, distribution, and abundance change between and within tissues. It is also increasingly clear that the ECM is remodeled in disease-specific patterns. The interactions between organ- or disease-specific ECM and resident cells are a subject of intense research and engineering. Precisely mapping the three-dimensional ECM structure across tissues and diseases is therefore a fundamental task. Here, we discuss in situ decellularization of tissues (ISDoT) as an essential tool to map the ECM supporting primary and metastatic tumors in experimental mice.

Key words Extracellular matrix, Cancer, Metastasis, Decellularization, High-resolution imaging

1 Introduction

The removal of cells from a tissue, resulting in a cell-free ECM scaffold, is termed decellularization [1]. Decellularization is extensively used to generate biomaterials that retain certain biological traits (e.g., protein composition). The multiple methods available to achieve decellularization have been extensively reviewed elsewhere [2]. Of interest here, perfusion decellularization of rodent organs [1, 3, 4] confirmed one fundamental notion: it is the ECM that sets and maintains organ shape and structure (Fig. 1). Crucially for ECM mapping, decellularization of murine tissue also revealed a pathological ECM acting as a scaffolding for tumors [1]. The method presented here, ISDoT, produces intact ECM scaffold for experimental exploration. Briefly, ISDoT requires surgery to access an organ or tissue, the catheterization, under a surgical microscope, of a blood vessel vascularizing it and the perfusion of the tissue with solutions that emulsify cell membranes and remove all cells, leaving an ECM scaffold that maintains its microscopic structure and

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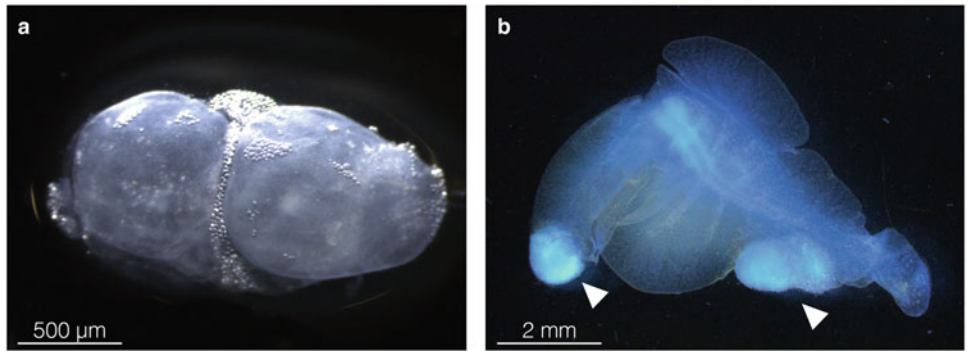

Fig. 1 In situ decellularization of organs generates ECM scaffolds. (**a**) Decellularized axillary lymph nodes. (**b**) Decellularized postcaval pulmonary lobe harboring lung metastases (white arrowheads). Notice that organ structure is preserved after all cells are removed from the tissue

chemical composition intact. This scaffold can be immunostained to mark ECM components for identification with fluorescent microscopy. Combining confocal microscopy with 2-photon microscopy associates second-generation harmonics imaging (i.e., fibrillar collagen) and elastin autofluorescence to immunostained components, opening the way to comprehensive ECM mapping.

ISDoT requires specialized surgical equipment (*see* Subheading 2 below), microsurgical training (*see* Subheading 3 below), and thorough familiarity with 2-photon-cum-confocal imaging and image processing.

The aim of this particular ISDoT application is to decellularize a primary tumor located in the mammary fat pad.

2 Materials

See **Note 1**.

2.1 Surgery for Decellularization (Fig. 2)

1. CO_2 ventilation chamber for mouse euthanasia.
2. Silicone tubing (2-mm i.d. and 4 mm o.d.; Ole Dich, cat. no. 31399).
3. Polystyrene tray (~30 × 50 cm).
4. Luer-to-tubing male fittings (1/8 inch; World Precision Instruments, cat. no. 13158-100).
5. Needles (27 gauge; Microlance, BD, cat. no. 21018).
6. Syringes (1 mL; Plastipak; BD, cat. no. 3021001).
7. Syringes (10 mL; Plastipak; BD, cat. no. 3021110).
8. Hair clippers (Oster, cat. no. 76998-320-051).
9. Peristaltic pump (Ole Dich, cat. no. 110AC(R)20G75).
10. Dissection microscope (Leica, model no. S6 D).

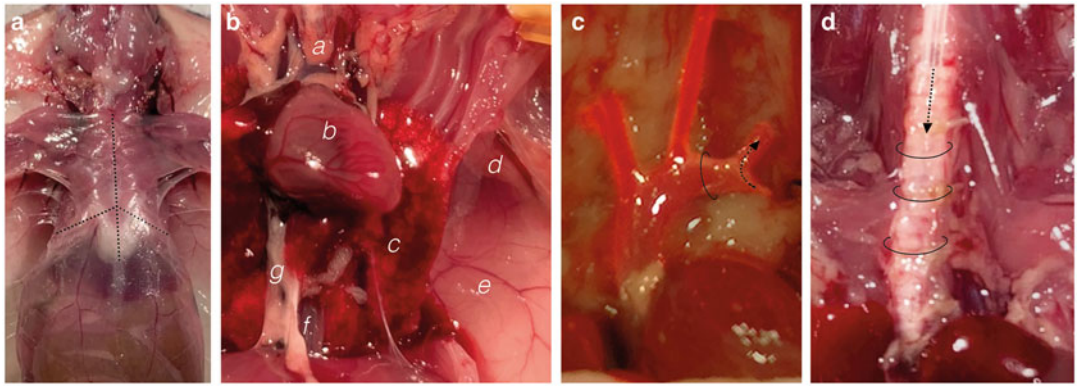

Fig. 2 Decellularization surgery. (**a**) Subcutaneous dissection of an experimental mouse. Frontal view. Dotted lines indicated the surgical access to the thorax. (**b**) After accessing the thorax and resecting the thymus, it is possible to identify (*a*) major vessels, (*b*) heart, (*c*) lungs, (*d*) axillary lymph nodes, (*e*) mammary fat pad, (*f*) thoracic aorta, and (*g*) cava vein. (**c**) Major vessel dissection. The aorta is ligated after the emergence of the left common carotid artery (smooth line). Retrograde flow, coming from a catheterized aorta (dotted arrow) is shunted towards the left subclavian artery irrigating the left mammary fat pad and the axillary lymph nodes. (**d**) Trachea catheterization to perfuse lungs (dotted arrow). The catheter is secured by microsuturing the trachea (smooth lines)

11. Electrosurgery unit with monopolar and bipolar electrodes (KLS Martin Minicutter).
12. Microvascular clamps (7, 11, and 16 mm; Fine Science Tools, cat. Nos. 00396-01, 00398-02, and 00400-03).
13. Clamp-applying forceps (Fine Science Tools, cat. no. 00072-14).
14. Double-ended microspatula (Fine Science Tools, cat. no. 10091-12).
15. Castroviejo microneedle holder (Fine Science Tools, cat. no. 12061-01).
16. Micro-spring scissors (Vannas, curved; Fine Science Tools, cat. no. 15001-08).
17. Dumont microforceps with 45° tips (two; Fine Science Tools, cat. no. 11251-35).
18. Dumont microforceps (two; Fine Science Tools, cat. no. 11252-20).
19. Microforceps with ringed tips (Aesculap, cat. no. FM571R).
20. Halsey needle holder (Fine Science Tools, cat. no. 12500-12).
21. Mayo scissors (Fine Science Tools, cat. no. 14110-15).
22. Metzenbaum scissors (Fine Science Tools, cat. no. 14017-14).
23. Serrated scissors (Fine Science Tools, cat. no. 14958-09).
24. Tendon scissors (Walton; Fine Science Tools, cat. no. 14077-09).

25. Adson forceps with teeth (Fine Science Tools, cat. no. 11027-12).
26. Adson forceps (Fine Science Tools, cat. no. 11006-12)

    6–0 suture, triangular section needle (Vicryl; Ethicon, cat. no. 6301124)

    9–0 micro-suture (BBraun, cat. no. G1048611).
27. Intravenous 24-gauge catheter (Insyte; BD, cat. no. 381512).
28. Intravenous 26-gauge catheter (Terumo; Surflo-W, cat. no. SR + DM2619WX).
29. Molt Periostotome (Aesculap, cat. no. D0543R).
30. Spatula (Freer-Yasargil; Aesculap, cat. no. OL166R).
31. Paper towels (sterile) or surgical napkin.

2.2 For Tissue Preparation and Immunostaining

1. Scalpel (sterile, type 23; Swann Morton, cat. no. 0510).
2. Dumont forceps (no. 5; Fine Science Tools, cat. no. 11252-20).
3. Petri dish (Nunclon Delta Surface; Thermo Fisher Scientific, cat. no. 150350).
4. Conical-bottom tube (15 mL; Greiner Bio-One, cat. no. GR-188271).
5. CryoTube vials (Thermo Fisher Scientific, cat. no. 377267).
6. Rocking table (Duomax 1030; Heidolph, cat. no. 543-32210-00).
7. Aluminum foil.

2.3 For Imaging

1. Glass-bottom culture dishes (35-mm Petri dish with 14-mm microwells and no. 1.0 cover glass (0.13–0.16 mm); MatTek, cat. no. P35G-1.0-14-C).
2. Microscope (inverted multiphoton microscope; Leica, model no. SP5-X MP).
3. Microscope mount for circular dishes.
4. Fluorescence light source (Leica, model no. EL6000).
5. White-light laser (WLL; Leica).
6. Two-photon Ti–sapphire laser (Spectra-physics, Mai Tai DeepSee model).
7. Detectors (hybrid detector [Leica, HyD S model] and photomultiplier tubes (PMTs; Leica)).
8. Objective (lambda blue, 20×, 0.70 numerical aperture [NA] IMM UV; Leica, HCX PL APO model).
9. Post-acquisition image processing software (Image J/Fiji: https://imagej.net/Fiji).

2.4 Reagents (See Note 2)

1. Deionized water (Milli-Q IQ 7000, Ultrapure lab water system; Merck, cat. no. ZIQ7000T0).
2. 70% ethanol (absolute alcohol 99.9%; plum, cat. no. 1680766); absolute alcohol must be adjusted to 70% (vol/vol) using deionized water.
3. PBS (pH 7.4, 10×, Gibco; Thermo Fisher Scientific, cat. no. 70011044).
4. Sodium deoxycholate (DOC; Sigma-Aldrich, cat. no. D6750-100G). DOC is harmful if swallowed. Wear protective rubber gloves and work in a fume hood.
5. Sodium dodecyl sulfate (Sigma-Aldrich, cat. no. L3771). SDS is hazardous. Avoid contact with skin or eyes and avoid swallowing. Wear protective rubber gloves and work in a fume hood.
6. Penicillin–streptomycin (Gibco; Thermo Fisher Scientific, cat. no. 15140122).
7. Sodium azide (Sigma-Aldrich, cat. no. 08591-1ML-F). Sodium azide is hazardous to the environment, especially to water organisms. Work in a fume hood and dispose of waste according to your institution's guidelines.

2.5 Immunostaining

1. Deionized water (Milli-Q IQ 7000, Ultrapure lab water system; Merck, cat. no. ZIQ7000T0).
2. Serum (normal donkey serum; Jackson ImmunoResearch, cat. no. 017-000-121).
3. Bovine Serum Albumin bovine (fraction V, standard grade, lyophilized; Serva, cat. no. 11930.03).
4. Tween 20 (Sigma-Aldrich, cat. no. P9416-50ML).
5. PBS (pH 7.4, 10×, Gibco; Thermo Fisher Scientific, cat. no. 70011044).
6. Primary antibodies (Table 1).

2.6 Secondary Antibodies

1. Alexa Fluor 488 donkey anti-goat IgG (H + L) (cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-11055, RRID:AB_2534102).
2. Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21206, RRID:AB_2535792).
3. Alexa Fluor 488 donkey anti-rat IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21208, RRID:AB_2535794).

Table 1
Troubleshooting table [3]

Problem	Possible reason	Solution	Procedure
Catheterized vessel is teared by catheter or needle	Catheter or needle insertion is not carefully performed	(i) Pull the catheter until it gets back into the vessel (and pull the needle out to avoid penetrating the vessel anew); try to push it in beyond the tear and place a suture stitch medial to the tear to avoid backflow. If the tear is near the target organ, or it is impossible to get the catheter into the vessel, start a new operation (ii) When catheterizing the abdominal aorta, first introduce the catheter only 1 cm below the diaphragm, then complete the surgical access to the abdomen, expose the aorta, and introduce the catheter carefully, watching its progress inside the artery, helping it with forces if necessary, to avoid tearing the aorta	Surgical procedure
Vessel perforation	While suturing the catheter for the abdominal operations, the needle perforates the cava vein	Get around both aorta and cava vein in one stitch to avoid perforation	Surgical procedure
Remaining aorta is not long enough to be catheterized	The mouse is sectioned too low	Section the mouse so there is at least 1 cm of aorta exposed to facilitate catheterization. If not possible, start a new operation	Surgical procedure
Perfusion is inefficient	The feeding vessel is blocked	Dissect the common carotid until its bifurcation and ligate the internal carotid (the branch emerging posteriorly) to focus the flow on the external carotid	Surgical procedure

(continued)

Table 1
(continued)

Problem	Possible reason	Solution	Procedure
Catheter perforates the portal vein	Wrong handling of the catheter	Reinsert the catheter back into the portal vein and suture above the perforation to stop backflow	Surgical procedure
The target tissue is not perfused	Vessels are collapsed	(i) Place the mouse under the surgical microscope. Try to perfuse the tissue using a 1-mL syringe and detect points of leakage. Clamp the leakage points (ii) If there is no leakage, reconnect the tissue to the perfusion pump and increase the flow to 0.4 mL/min to open collapsed vessels	Decellularization
Organ is fractured by the perfusion	Flow speed is too high	Lower the flow speed	Decellularization
Organ is not decellularized	(i) Catheter is not properly placed and secured (ii) Flow speed is insufficient (iii) Sectioned vessels allow reagents to escape (iv) Reagents are not freshly prepared	(i) After completing the surgical procedure, verify the catheter placement, stitch tightness, organ perfusion, and reagent retention. Replace the catheter if it is not correctly placed and make additional tight stitches to secure and provide correct reagent flow into the organs (ii) We use a flow speed of 0.2 mL/min. Lower speeds may not guarantee whole organ perfusion (iii) Verify organ perfusion under the microscope and look for sectioned vessels, or vessels that are perfused but lead to tissues outside the area of interest (e.g., the internal thoracic arteries). Cauterize the vessels (iv) Prepare fresh reagents for every round of decellularization	Decellularization

(continued)

Table 1
(continued)

Problem	Possible reason	Solution	Procedure
Organ is incompletely decellularized	(i) Decellularization efficacy can vary due to anatomical differences in vascular structures and other surgical problems (ii) Catheter has been removed	(i) Detect nuclear DNA using two-photon excitation by tuning the laser to 950 nm and detecting 405–445 nm. If nuclear DNA is detected, start a new operation depending on the scientific question (ii) During decellularization, tissues will expand because of the perfusion. This change could dislodge the catheter and leave the area of interest incompletely decellularized. To prevent this problem, add tight securing stitches, attaching the catheterized vessel to the underlying tissue	Decellularization
Tumors are not decellularized	Tumor size is excessive	Tumors have an abnormal vasculature. When decellularizing tumors with a diameter greater than 1 cm, the vasculature may not reach all tumor areas. Optimize the cancer model to decellularize tumors smaller than 1 cm. Alternatively, increase flow speed	Decellularization
Organs are colonized by microorganisms in storage	Imperfect sterility during the decellularization procedure	All surgical materials must be autoclaved before the procedure and all materials should be sterile. All materials or instruments that are not amenable to sterilization (e.g., the dissection microscope) must be disinfected before use. To minimize the risk of microbial colonization, decellularization can be carried out inside a laminar flow hood. To prevent colonization of the decellularized tissue by microorganisms, place the	Storage

(continued)

Table 1
(continued)

Problem	Possible reason	Solution	Procedure
		tissues in the storing solution (penicillin/streptomycin [1% vol/vol] and sodium azide at a final concentration of 0.3 μM in deionized water)	
Images are moving/drifting during z-stack acquisition	Tissue is floating and not making contact to the bottom of the glass bottom dish because too much water has been added to the dish	Try first to aspirate the liquid inside the dish until the sample is stable. Alternatively, remove the tissue completely from the dish using the forceps and suck out the liquid with a pipette. Then, remount the tissue adding less water	Microscopy
No antibody signal	(i) One of the antibodies is not working (ii) The protein is not present in the tissue (iii) The signal is very weak (iv) You are looking at a tissue region without signal (v) The tissue is not under the light source (vi) Tissue has dried (vii) The secondary antibody has a wavelength which is beyond the scope of the microscope fluorescence	(i) Choose another antibody, primary or secondary (ii) Test another tissue type (iii) Adjust laser power and exposure time, but be careful not to amplify autofluorescence signal instead (iv) Flip the tissue to look at another area avoiding thick surfaces. Or consider slicing the tissue to expose new areas (v) Move the glass bottom dish (vi) Repeat experiment. Check that the right combination of primary and secondary antibodies has been used with the right fluorophores (vii) Test if the signal can be detected with another laser source and with another wavelength	Microscopy
Excessive fluorescence quenching/photobleaching	Excessive light exposure (during acquisition)	(i) Reduce laser power and adjust parameters that reduce laser exposure: increase spacing between z-stacks, reduce number of images/stacks or reduce pixel numbers, reduce pixel dwell time, and reduce averaging	Image acquisition

(continued)

Table 1
(continued)

Problem	Possible reason	Solution	Procedure
		(ii) Consider that your primary antibody is not suitable for the purpose (iii) Consider increasing the concentration of the antibodies during staining (iv) Consider exciting the fluorophore with two-photon excitation rather than 1-photon excitation	
Channel bleeding while testing multiple antibodies using simultaneous acquisition	(i) The signal in one detector is mistakenly originating from another antibody/fluorophore that is sending out a signal that "bleeds" into adjacent channels and is detected here as a false positive (ii) You are using too much laser power (iii) Your primary antibody is strong	(i) To test if light from the fluorophore is bleeding into the wrong channels, activate the laser at one wavelength, and open all the detectors at the other channels too. If there are signals in the wrong channels, you have a bleed-through problem (ii) Try reducing the laser power to a level where there is only signal in the correct channel. Or repeat test with other secondary antibodies with a larger distance between the wavelengths (iii) The strongest primary antibody should be excited by the longest wavelength (iv) Use sequential rather than simultaneous channel acquisition (only one fluorophore at a time)	Image acquisition
No signal (apart from SHG)	There is no autofluorescence	You can trust the signal in your positive control	Negative control
A weak signal	The autofluorescence of your sample is excited by the laser light	(i) Reduce the laser power to a level where no signal is detected and repeat the tests in the positive control (ii) Try to adjust the gain or the threshold of the background (iii) Change to a different secondary antibody	Negative control

(continued)

Table 1
(continued)

Problem	Possible reason	Solution	Procedure
Strong signal	(i) The autofluorescence of your sample is strongly excited by the laser emission (i.e., elastin) (ii) You mistakenly added the primary antibody to the negative control	(i) Change to a secondary antibody with a different wavelength where little to no autofluorescence is present (ii) Repeat the immunostaining without adding a primary antibody to your negative control	Negative control

4. Alexa Fluor 555 donkey anti-mouse IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-31570, RRID:AB_2536180).
5. Alexa Fluor 555 donkey anti-rabbit IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-31572, RRID:AB_162543).
6. Alexa Fluor 594 donkey anti-mouse IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21203, RRID:AB_2535789).
7. Alexa Fluor 594 donkey anti-rat IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21209, RRID:AB_2535795).
8. Alexa Fluor 594 donkey anti-rabbit IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21207, RRID:AB_141637).
9. Alexa Fluor 594 donkey anti-goat IgG (H + L) (cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-11058, RRID:AB_2534105).
10. Alexa Fluor 594 donkey anti-guinea pig IgG (H + L) (Jackson ImmunoResearch Labs, cat. no. 706–585-148, RRID: AB_2340474).
11. Alexa Fluor 647 donkey anti-goat IgG (H + L) (cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-21447, RRID:AB_2535864).
12. Alexa Fluor 647 donkey anti-rabbit IgG (H + L) (highly cross-adsorbed secondary antibody; Thermo Fisher Scientific, cat. no. A-31573, RRID:AB_2536183).

3 Methods

3.1 Setup

All solutions must be prepared fresh each time.

1. *Decellularization solutions*: Add 0.5% (wt/vol) DOC to deionized water.

   Add 0.1% (wt/vol) SDS to deionized water.
2. *Blocking solution*: Add 3% (wt/vol) BSA to 1× PBS. Choose the serum depending on the source of the secondary antibody (goat or donkey in this protocol) and add 6% (vol/vol) serum. Mix for 30 min on a shaker.
3. Primary antibody solution: Prepare the primary antibody solution by thawing and adding 3% (vol/vol) serum to 1× PBS. Prepare the desired concentration of primary antibody by adding it to the solution.
4. Negative-control solution: Prepare the negative-control solution by thawing and adding 3% (vol/vol) serum to 1× PBS.
5. *Washing solution*: Prepare the washing solution by adding 0.05% (vol/vol) Tween 20 to 1× PBS.
6. *Secondary antibody solution*: Prepare the secondary antibody solution by thawing and adding 3% (vol/vol) serum to 1× PBS. Select the secondary antibodies (described below) and add them in a 1:1000 (vol/vol) concentration. Plan which primary and secondary antibodies you will combine. The primary antibody is produced in a host animal, and the secondary antibody you choose has to react with the animal species used to produce the primary antibody. When using multiple primary antibodies simultaneously, make sure that they are all produced in different host species.
7. *Storing solution*: Add penicillin–streptomycin (1% [vol/vol]) and sodium azide at a final concentration of 0.3 μM to deionized water. Store at 4 °C for up to 6 months.

3.1.1 Two-Photon Microscopy Setup

We imaged immunostained decellularized tissues using an inverted microscope connected to a Ti–sapphire laser (tunable wavelength: 690–1040 nm) and a supercontinuum WLL and a HCX PL APO, lambda blue, 20×, 0.70 NA, IMM, UV objective. Fibrillar collagen was detected by means of SHG using two-photon excitation at 892 nm, and the emitted light between 426 and 446 nm was detected using a hybrid detector. Immunostainings using Alexa Fluor 488 were simultaneously excited using the 892-nm two-photon excitation and the emitted light was collected between 505 and 550 nm using a PMT detector. Decellularized tissue stained with Alexa Fluor 555, Alexa Fluor 594, and Alexa Fluor 647 were all sequentially excited with a supercontinuum WLL, and the emitted light was detected using the hybrid detector. All images are from

backscattered light and captured with a resolution of minimum 1024 × 1024 pixels at 100–200 Hz.

3.2 Procedure

This section describes the preparation and surgical procedures needed to decellularize a mammary fat pad primary breast tumor, lymphatic, and pulmonary metastases. All experiments must follow governmental and institutional regulations.

3.2.1 Preparation

1. Kill mice with CO_2-induced hypoxia. To do this, place the mouse in a ventilation chamber where the air is gradually substituted by 20% CO_2 for 2 min and then increased to 100% CO_2.
2. Shave the front and back of the mouse using the clippers and disinfect the skin with 70% ethanol.
3. Stretch and pin the mouse to the surgical table, running the needles through the forelimbs, hind limbs, tail, and nose. Using the Mayo scissors and the Adson forceps, make skin incisions, two laterally in the low abdomen and one from the low abdomen to the mandible, and dissect the skin to expose the peritoneal wall, the thorax, and the neck.

3.2.2 Surgery

See **Note 3**

1. Using Dumont microforceps and micro-spring scissors, section the pectoralis muscles at the height of the xyphoid process to reveal the rib cage and intercostal muscles. Using the serrated scissors, cut through the sixth intercostal space on the left and right sides. Section the sternum horizontally to create an opening into the thorax. Avoid cutting the lungs when sectioning through the intercostal muscles. Irrigate the tissues regularly with PBS.
2. Using the serrated scissors, section the sternum along its midline axis (sternotomy). Elevate and pin the thoracic walls of each side to reveal the contents of the thorax.
3. Using the bipolar electrode, cauterize the vascular bundles running along the internal face of the sternum and the cava vein above its entrance into the abdomen, leaving it to be catheterized later (alternatively, these vessels can also be clamped using the clamp-applying forceps and microvascular clamps or ligated with micro-suture and sectioned).
4. Using the Adson forceps and the Mayo scissors, create a tunnel between the lumbar spine and the skin. The tunnel must be spacious enough to allow the passage of the scissors. Section the body of the mouse into two separate halves along an axis transecting the aorta (making an opening into it) and spine; cut the aorta, spine, and ribs skin first, passing through the tunnel, and then cut the skin inferiorly to the primary breast tumor.

3.2.3 Surgery to Decellularize Tissues in the Territory of the Left Subclavian Artery and the Cardiopulmonary Complex (i.e., Primary Tumor, Axillary Metastasis, Pulmonary Metastasis)

1. Detach the thymus using the ring-tipped microforceps and the Dumont forceps to reveal the emergence of the aorta, aortic arch, and major vessels. Take care not to disrupt the underlying tissues, particularly the veins under the thymus.
2. Using the ring-tipped microforceps and Dumont microforceps, expose the left subclavian artery by delicately detaching the left brachiocephalic vein and liberate the aortic arch around its emergence from the underlying tissue.
3. Using the Dumont microforceps and the clamp-applying forceps, clamp the aortic arch after the emergence of the left common carotid artery. Alternatively, the aorta can be ligated with a 9–0 micro-stitch.
4. Insert a 24-gauge catheter into the aorta through the opening previously made through the aorta (*see* **step 7**). Push the catheter until it reaches the emergence of the left subclavian artery and then retreat ~1 cm. During each catheter insertion, withdraw the needle as soon as the catheter enters the vessel to avoid punctures or rips that could lead to reagent escape and incomplete decellularization.
5. Using the Dumont microforceps, the Castroviejo microneedle holder, and the 9–0 suture, place a stitch 5 mm below the tip of the catheter and two additional stitches 1 cm below the first stitch. Make a final stitch, using the Halsey needle holder, Adson forceps, and 6–0 suture, around the spine and the catheter to avoid its dislodgment (Fig. 3b). Each stitch must be secured tightly to avoid reagent backflow and catheter dislodgement; be careful not to break the micro-suture thread. A 26-gauge catheter can also be used, as long as the securing stitches are sufficient.
6. Insert a 26-gauge catheter into the descending thoracic cava vein and push its tip until it reaches the entrance to the right atrium. Secure it with three 9–0 stitches. Avoid pushing the venous catheter into the heart. This will damage the atrium.
7. Using the micro-scissors, cut the median cricothyroid ligaments and muscles, opening an entrance to the trachea. Insert a 24-G catheter in the trachea advancing until the catheter is stopped by trachea bifurcation. Secure with three 6–0 stitches. Avoid tearing or perforating the trachea.

3.3 Decellularization

1. Place the catheterized mouse in a Styrofoam tray and fix the catheter to the tray with needles. Connect the catheters to the silicone tubing with a Luer connector bit. The silicone tubing should be connected to the peristaltic pump and a reservoir containing the reagent. Set the peristaltic pump at a flow output of 200 μL/min (288 mL/24 h).
2. Perfuse with deionized H_2O for 2 h.

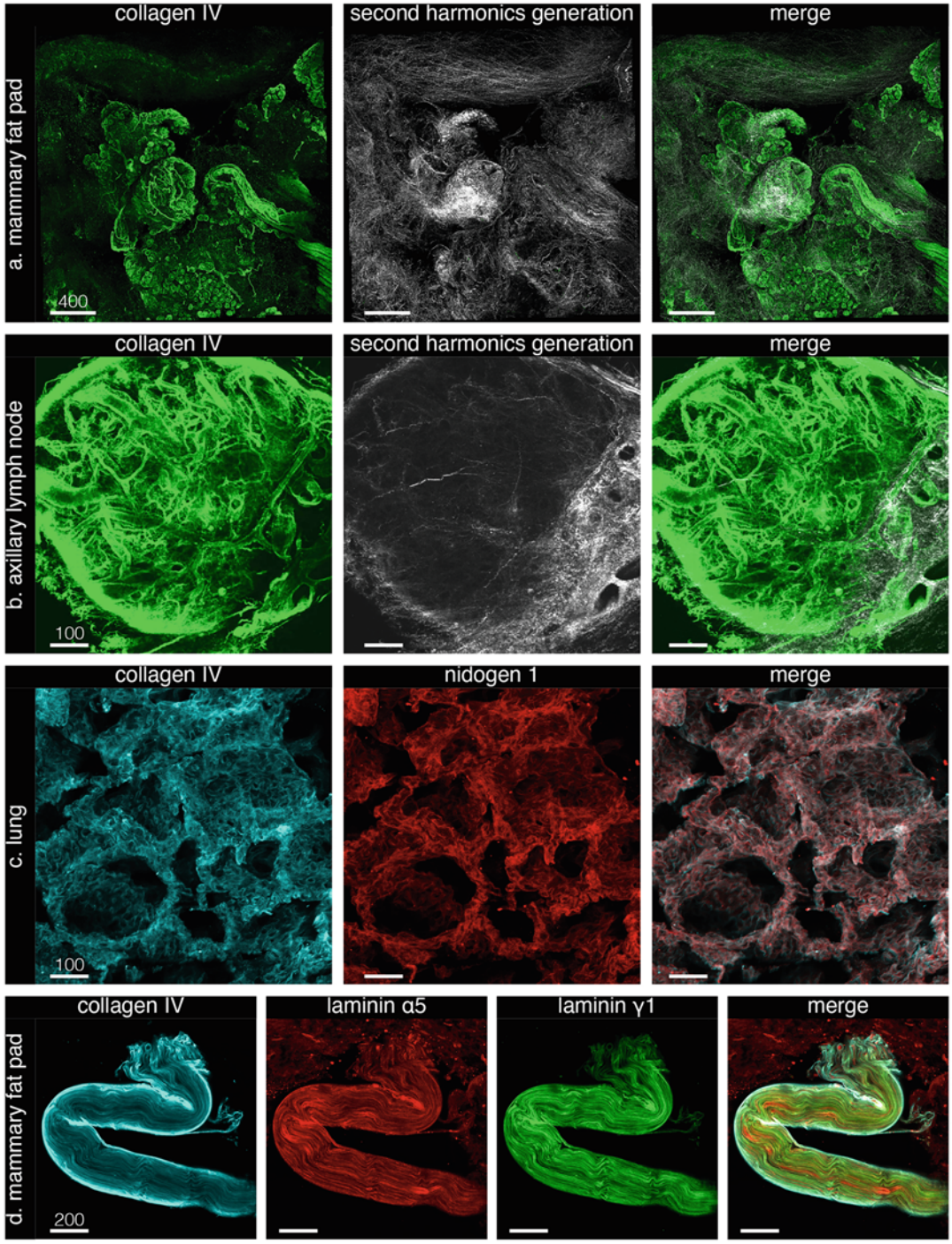

Fig. 3 ECM imaging. (**a**) Tiled z-stack reconstruction of a mouse mammary fat pad harboring a primary breast cancer tumor. (**b**) Z-stack reconstruction of a metastatic axillary lymph node. (**c**) Z-stack reconstruction of the mouse lung parenchyma. (**d**) Z-stack reconstruction of a peripheral nerve trunk in a mouse mammary fat pad. All scale bars in microns

3. Perfuse with DOC solution overnight (18 h).
4. Perfuse with SDS solution for 6–18 h.
5. Switch the perfusion to deionized water and perfuse for 18 h.
6. Use the Dumont microforceps and the micro-spring scissors to collect the decellularized tissues and place them in a CryoTube with storing solution. At this point, the decellularized tissue can alternatively be frozen for downstream chemical analysis such as tandem mass spectrometry (MS/MS). Unused and unnecessary mouse tissues, used reagents, and equipment must be disposed according to the user's animal facility regulations. The tissue can be stored for up to 6 months at +4 °C.

3.3.1 Preparing Decellularized Tissues for Immunostaining

1. Prepare the blocking solution. Add enough to cover each tissue segment inside a CryoTube and place the samples on a rocking table overnight (~15 r.p.m. at room temperature for all experiments). Be very careful not to damage the tissue segments with the pipette tip during liquid changes. Avoid tissue dehydration between liquid changes by proceeding quickly.
2. Prepare the primary antibody solution and negative-control solution. Discard the blocking solution from the tissue container and add the primary antibody solution to the test tissues and negative-control solution to the negative controls. Add enough solution to cover the samples completely. Label the tubes and place them on the rocking table overnight. We recommend an incubation of 20–24 h.
3. Prepare the washing solution. Discard the primary antibody solution in the tissue container and add washing solution to cover the samples completely. Leave on the rocking table for 1 h; discard the washing solution and add fresh washing solution. Repeat until you have carried out five washing cycles.
4. Prepare the secondary antibody solutions. Discard the washing solution from the tissue and add secondary antibody solutions to the samples, including the negative-control samples. Protect the tubes from light by wrapping them in aluminum foil and place them on the rocking table overnight.
5. Discard the solution from the tissue container and add washing solution. Leave on the rocking table for 1 h, remove the washing solution, and add fresh washing solution. Repeat until you have carried out three washing cycles and then fill the containers with storage solution. The tissue can be stored for up to 6 months at +4 °C; however, we recommend proceeding to the imaging step as soon as possible to avoid fluorescence decay.

3.4 Preparation for Imaging of the Decellularized Tissues with a Multiphoton/ Confocal Fluorescence Microscope

1. Using the ring-tipped microforceps, delicately transfer the decellularized tissue to a glass-bottom dish. Place the tissue with the surface you wish to image facing down on the glass bottom. Add one or two droplets of extra storage solution to the tissue with a pipette to avoid tissue drying. Make sure that the tissue is not floating around in the liquid and that it is sticking to the glass bottom.
2. Place a droplet of immersion liquid (for the objective used here, water) on the cleaned objective, and place the glass-bottom dish on the circular frame mount holder. Elevate the lens until the water droplet makes contact with the bottom of the dish. Turn on the fluorescence light source (bulb) and focus the sample. Navigate and position the area you wish to image. Turn off the fluorescent light (bulb) before moving to image acquisition using laser light.

3.5 Image Acquisition (Fig. 3)

Switch to the computer control and go live with the white-light or two-photon laser, depending on which you are using. If you are using both, start with the two-photon laser. Reduce the laser power to avoid fluorescence quenching. Select and adjust detectors (hybrid and PMTs) to accommodate your chosen fluorophores. Choose simultaneous or sequential image acquisitions. Adjust laser wavelengths, power, gain, and offset. Adjust pinhole, pixel size, pixel dwell time, averaging, and the zoom. Remember to verify that the negative control gives no signal in your detectors. Set the z-dimensions by scrolling on the computer z-controller and define the start and end points. The numbers of images given within a volume (z-stack) are chosen ad hoc to accommodate the purpose. Acquire images. If you intend to image the same sample for longer periods of time, make sure that sufficient storage solution is hydrating the sample at all times and that the immersion liquid on the objective is not drying out (*see* **Notes 4–6**).

4 Notes

1. All surgical procedures and sample storage must be done under sterile conditions.
2. All solutions must be prepared fresh each time.
3. From point **step 1**, Subheading 3.2.2, dissect under the dissection microscope.
4. The imaged tissue can be stored for up to 6 months at +4 °C, but a reduction in fluorescence signal over time may take place.
5. Detailed videos of ISDoT surgery are available on references [1] and [5].
6. The analysis and quantification of 3D imaging falls outside the scope covered by this protocol.

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Chapter 6

Monitoring Cell Cytoskeleton Variations upon Piezoelectric Stimulation: Implications for the Immune System

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Abstract

Piezoelectric stimulation can have a significant impact on different cellular functions with possible applications in several fields, such as regenerative medicine, cancer therapy, and immunoregulation. For example, piezoelectric stimulation has been shown to modulate cytoskeleton variations: the implications of this effect range from the regulation of migration and invasion of cancer cells to the activation of pro- or anti-inflammatory phenotypes in immune cells. In this chapter, we will present different methodologies to evaluate cytoskeleton variations, focusing on modifications on *f*-/*g*-actin ratio and on the migration and invasion ability of tumor cells.

Key words Piezoelectric stimulation, Cytoskeleton, Migration assay, Invasion assay, *f*-/*g*-actin ratio

1 Introduction

Piezoelectric stimulation of cells mediated by ultrasound (US) activation of piezoelectric nanomaterials is gaining attention in the latest years due to its effects on different cellular functions, including morphology, proliferation, migration, differentiation, or inhibition of cell cycle progression [1]. This kind of stimulation is less invasive with respect to other approaches, owing to the wireless US activation of piezoelectric materials that can, in turn, induce locally, at the site of interest, the electric cue. Piezoelectric stimulation holds high potentialities in different therapeutic areas, such as neuro-modulation, regenerative medicine, and cancer therapy; nevertheless, a lot of effort must be addressed into a more thorough characterization of the actual biological effects. In fact, despite the technological interest, the mechanism behind piezoelectric activation of cells has not been completely unraveled yet, mainly because it depends on many factors, including the stimulation protocol, the cell/piezoelectric material interaction, and the cell type [1]. For example, several studies have reported that piezoelectric

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stimulation has beneficial effects on the proliferation of fibroblasts and macrophages [2, 3], for the differentiation of neuroblastoma and osteosarcoma cells [4, 5], and for the adhesion and chondrogenesis of mesenchymal stem cells [6, 7]. On the other hand, piezoelectric stimulation of different kinds of tumors, such as glioblastoma multiforme and breast cancer, has been demonstrated to induce apoptosis and to inhibit cell growth [8, 9]. Piezoelectric stimulation was also shown to regulate the immunological response, by activating the pro-inflammatory (M1) or anti-inflammatory phenotype (M2) of macrophages, depending on the stimulation parameters [10, 11].

One common effect observed in several studies after piezoelectric stimulation concerns modifications at the level of the cell cytoskeleton and, in particular, on the ratio between globular (*g*-actin) and filamentous actin (*f*-actin) [12]. The basic architecture of the cytoskeleton is composed by three main filamentous entities (actin, microtubules, and intermediate filaments) that maintain cell shape and modulate fundamental dynamic cellular functions, including cell migration and the ability to respond to external cues [13]. In particular, *f*-actin is involved in preserving cell morphology and in forming membrane protrusion (lamellipodia), thus playing a key role in endocytosis, cytokinesis, adhesion, and migration [13]. Since *f*-actin accumulation in leading edges of cells has an important role during cell migration, the *f*-/*g*-actin ratio can give important information on the variation of cell motility and migration capability following a specific treatment, especially in highly invasive tumors; the lower the *f*-/*g*-actin ratio, the lower the cells tendency to migrate in neighboring areas. In one of our recent studies, we have demonstrated in vitro that the treatment of a glioblastoma multiforme model (T98G cells) with polymeric piezoelectric nanoparticles based on poly(vinylidene fluoride-trifluoro ethylene) (P(VDF-TrFE)), loaded with the drug nutlin-3a, was able to reduce the *f*-/*g*-actin ratio only after application of US [9]. This suggests that the piezoelectric stimulation might have an impact on the arrangement of the cytoskeleton of tumor cells, reducing their migration and invasion activity. The effect of piezoelectric stimulation on the *f*-/*g*-actin ratio and on cytoskeleton was observed also in other cell types, such as fibroblasts [12], and in different cells of the immune system. This might have important repercussions in immunoregulation and/or immunotherapy, since it has been demonstrated that cytoskeleton plays an important role in the activation of immune cells, in the regulation of functions involved in pathogen detection, and in the host defense mechanism [14]. Actin is involved in several functions of the immune system, such as locomotion, cell/cell interaction, endocytosis, phagocytosis, or cell morphology [13]. For example, an increase in *f*-actin was shown to be correlated to an increase of the phagocytic activity of neutrophils [15] and of the nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase activity of microglia cells [16] and to have a role in natural killer cell activation [17]. Moreover, piezoelectric stimulation has been demonstrated to affect immune cell activation: for example, the M1 state of macrophages has been activated by ultrasound stimulation on a PVDF membrane [10].

Due to the crucial role of cytoskeleton in several cellular functions and in the migration/invasion ability of tumor cells, in this chapter we provide a detailed description about how to perform the analysis of the *f*-/*g*-actin ratio and migration and invasion assays following piezoelectric stimulation of cells, according to the methodologies routinely used in our laboratories. The described tests refer to experiments performed on glioblastoma cells, stimulated by US activation of P(VDF-TrFE) nanoparticles; nevertheless, due to their versatility, they can be easily translated to other cell types and/or other kinds of piezoelectric stimuli.

2 Materials

All solutions were prepared using MilliQ water at room temperature unless otherwise specified.

2.1 Preparation of the Particle Dispersion

1. Nutlin-3a (Nut, Sigma-Aldrich, SML0580). Store at −20 °C.
2. PNPs: apolipoprotein E-derived peptide (GenScript, sequence CWGLRKLRKRLLR) functionalized P(VDF-TrFE) (45:65, Piezotech) nanoparticles (*see* **Note 1**). Store at 4 °C.
3. Nut-PNPs: nutlin-3a-loaded PNPs. Store at 4 °C.

2.2 Cell Culture

1. Glioblastoma cell line (T98G, ATCC® CRL-1690™).
2. Dulbecco's modified Eagle's medium (Sigma-Aldrich, D6429) supplemented with 10% (v/v) fetal bovine serum (Gibco, 16000044), 100 U/mL penicillin-streptomycin (Gibco, 15140122), and 2 mM l-glutamine (Gibco, 25030081) (*see* **Note 2**). Store at 4 °C.
3. Dulbecco's phosphate-buffered saline (PBS, Sigma-Aldrich, D8537) without calcium chloride (Ca^{2+}) and magnesium chloride (Mg^{2+}).
4. Trypsin/EDTA (Gibco, 25200056). Store at 4 °C.
5. Ethanol.
6. Bleach.
7. Cell culture flasks (T75) and plates (96-well plate, 12-well plate).
8. 5-, 10-, and 25-mL serological pipettes.
9. Sterile 15-mL conical tubes.

10. Hemocytometer.
11. Glass coverslip.
12. 1.5–2-mL plastic tubes.
13. 10-, 200-, and 1000-μL pipettes and sterile tips.
14. Waste containers.

2.3 Scratch/ Migration Assay

1. Culture insert 2-well systems (Ibidi, 80209).
2. 12-well plates.
3. Sterile tweezer.
4. PBS (Sigma-Aldrich, D8662) with Ca^{2+} and Mg^{2+}.
5. Calcein (Invitrogen, C3099). Store at −20 °C.

2.4 f-/g-Actin Ratio

1. 4% (w/v) paraformaldehyde (PFA, Sigma-Aldrich, 158127) solution: Add 4 g of PFA to 80 mL of PBS and heat the solution on a stir plate at 60 °C in a ventilated hood. Add 1 mL of 1 M sodium hydroxide (NaOH, Sigma-Aldrich, 567530) and stir gently until the PFA is dissolved. Adjust pH to 7.4 with 1 M hydrochloride (HCl, Sigma Aldrich, 320331), and then adjust the final volume to 100 mL with PBS (*see* **Note 3**).
2. Culture insert 2-well systems (Ibidi, 80209).
3. 12-well plates.
4. Sterile tweezer.
5. PBS with Ca^{2+} and Mg^{2+}.
6. Triton X-100 (Sigma-Aldrich, T8787).
7. TRITC-phalloidin (Sigma-Aldrich, P1951). Store at −20 °C.
8. Deoxyribonuclease I conjugate (Invitrogen, D12371). Store at −20 °C.
9. Hoechst 33342 (Invitrogen, H1399). Store at −20 °C.

2.5 Trans-Well Invasion Assay (Abcam, ab235887)

1. Cell invasion chambers (24 wells, 8 μm).
2. Basement membrane matrix (collagen I). Store at −20 °C.
3. Wash buffer. Store at −20 °C.
4. Cell dissociation solution. Store at −20 °C.
5. Control invasion inducer. Store at −20 °C.
6. Cell dye. Store at −20 °C.
7. Tris–HCl buffer (Invitrogen, 15567027).
8. Cotton swabs.
9. 96-well clear-bottom white plates.

2.6 Equipment

1. Cell culture hood (MSC-Advantage™ Class II Biological Safety Cabinets, Thermo Fisher Scientific).
2. Epifluorescence microscope (Eclipse Ti, Nikon).
3. Incubator (Heracell™ 150i, Thermo Fisher Scientific) and CO_2 supplier (Nippon Gases, Italy).
4. Centrifuge (Hettich® Universal 320/320R centrifuge, Sigma Aldrich).
5. Water bath (Julabo® TW12, Sigma Aldrich).
6. Sonitron GTS Sonoporation System (ST-GTS, Nepagene) equipped with a plane wave transducer module (PW-1.0-6 mm, 6-mm-diameter tip, 1 MHz).
7. Confocal fluorescence microscope (C2 system Nikon).
8. Fluorescence plate reader (VICTOR X3, PerkinElmer).

2.7 Image and Statistical Analysis

1. ImageJ software with "Wound Healing" plug-in (https://imagej.nih.gov/ij/plugins/index.html).
2. NIS-Elements software (Nikon).
3. ANOVA parametric test followed by Tukey's HSD post-*hoc* test by using *R* software (https://www.r-project.org/).

3 Methods

3.1 Preparation of the Particle Dispersion

1. Prepare 5 mM Nut in dimethyl sulfoxide (DMSO) as a stock solution. Store at 4 °C.
2. Sonicate PNPs and Nut-PNPs for 2 min by using an output power of 8 W.
3. Prepare 500 μg/mL of PNPs and Nut-PNPs in complete media.

3.2 Cell Seeding

All solutions have to be pre-warmed at 37 °C in a water bath. Prior to start, the working area and all reagents have to be thoroughly disinfected using 70% (v/v) ethanol (*see* **Note 4**). In addition to routine cleaning, wipe the work surface with 70% (v/v) ethanol during work, in particular after any spillage.

1. Culture T98G cells in a T75 flask with 12 mL of complete medium at 37 °C in a 5% CO_2 atmosphere. The medium has to be changed every 2 days.
2. Split the cells when the culture reaches 90% confluency.
3. Discard the medium from the cell culture flask.
4. Wash cells using 5 mL PBS without Ca^{2+} and Mg^{2+} (*see* **Note 5**).
5. Remove PBS from the cell culture flask.

6. Add 3 mL of trypsin/EDTA to the flask to cover the cell layer.
7. Incubate the cells for 5 min at 37 °C (*see* **Note 6**).
8. Observe the cells under a phase-contrast microscope for checking detachment (*see* **Note 7**).
9. Add 6 mL of complete media (two volumes of trypsin/EDTA used for detachment) to stop trypsin/EDTA action.
10. Transfer the cells into a 15-mL conical tube and centrifuge at 600 *g* for 5 min (*see* **Note 8**).
11. Discard the supernatant.
12. Resuspend the cell pellet using 1 mL of complete media.
13. Determine the total cell number using a cell counter (hemocytometer).
14. Dilute the cell suspension to the cell seeding density required for each protocol below.

3.3 Migration Assay

1. Place culture insert 2-well systems (Ibidi, 80209) in a 12-well plate using a sterile tweezer.
2. Seed 70 μL of cell suspension ($5 \cdot 10^4$ cells/cm^2 seeding density) into each well (*see* **Note 9**).
3. Incubate for 24 h at 37 °C to allow cell attachment.
4. Discard media and add plain complete medium as control, 21.5 μM Nut, 500 μg/mL PNPs, or 500 μg/mL Nut-PNPs for 24 h.
5. After the incubation, gently remove the inserts by grabbing one corner using a sterile tweezer (*see* **Note 10**).
6. Rinse the cells using PBS with Ca^{2+} and Mg^{2+} to remove nonadherent cells and/or cell debris.
7. Stain the cultures using 1 μM calcein (prepared in PBS with Ca^{2+} and Mg^{2+}) for 15 min at 37 °C.
8. Wash the cells using PBS with Ca^{2+} and Mg^{2+}.
9. Image the gap between cells using a 4× objective (time 0 h).
10. Stimulate the cells by delivering US with 1 W/cm^2 intensity and 1 MHz frequency for 1 h/day for 2 days. Place the US probe tip on the plate in contact with the culture medium at a distance of 5 mm from the cells.
11. Image the gap between cells using a 4× objective (time 24 h). Representative images are shown in Fig. 1.
12. Analyze the images by using ImageJ software using the "Wound Healing" plug-in.

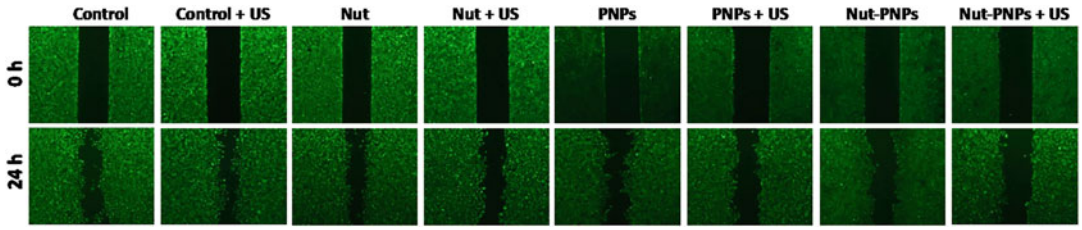

Fig. 1 Representative fluorescence images of T98G cells stained with calcein at $t = 0$ h and $t = 24$ h during the migration assay. (Reproduced from [9] with permission from Elsevier)

3.4 Actin Cytoskeleton Organization

1. Seed the cells and treat the cultures as already described in Subheading 3.3
2. Stimulate the cultures with US using the same parameters described above.
3. At the end of treatment, fix the cultures with 4% PFA at 4 °C for 20 min.
4. Rinse the cells using PBS with Ca^{2+} and Mg^{2+}, and then permeabilize with 0.1% (v/v) Triton X-100 solution for 15 min at room temperature.
5. Remove the solution, stain *f*-actin, *g*-actin, and nuclei with TRITC-phalloidin (1:200, v/v), deoxyribonuclease I conjugate (0.3 μM), and Hoechst (1:1000, v/v) at 37 °C for 45 min.
6. Wash the cells using PBS with Ca^{2+} and Mg^{2+}.
7. Acquire the images from the scratch region using a confocal fluorescence microscope.
8. Analyze *f*-/*g*-actin signal ratio with NIS-Elements software regarding red/green signals (Fig. 2).

3.5 Invasion Assay

All reagents were equilibrated to room temperature just prior to use and gently agitated to avoid precipitation.

1. Add 100 μL of collagen I to the filters to form a film.
2. Incubate the plate for 2 h at 37 °C (*see* **Note 11**).
3. Add 200 μL of a cell suspension ($2.3 \cdot 10^5$ T98G cells/chamber) in complete medium supplemented with 21.5 μM Nut, 500 μg/mL PNPs, or 500 μg/mL Nut-PNPs to each well of the upper chamber.
4. Under sterile conditions, disassemble the top chambers and add 600 μL of culture medium in the lower chambers.
5. Reassemble the top and the lower chambers (*see* **Note 12**).
6. After 12 h of incubation, replace the lower chamber medium with a control invasion-inducer medium (1:10, v/v).
7. Stimulate the cultures with US as previously described.

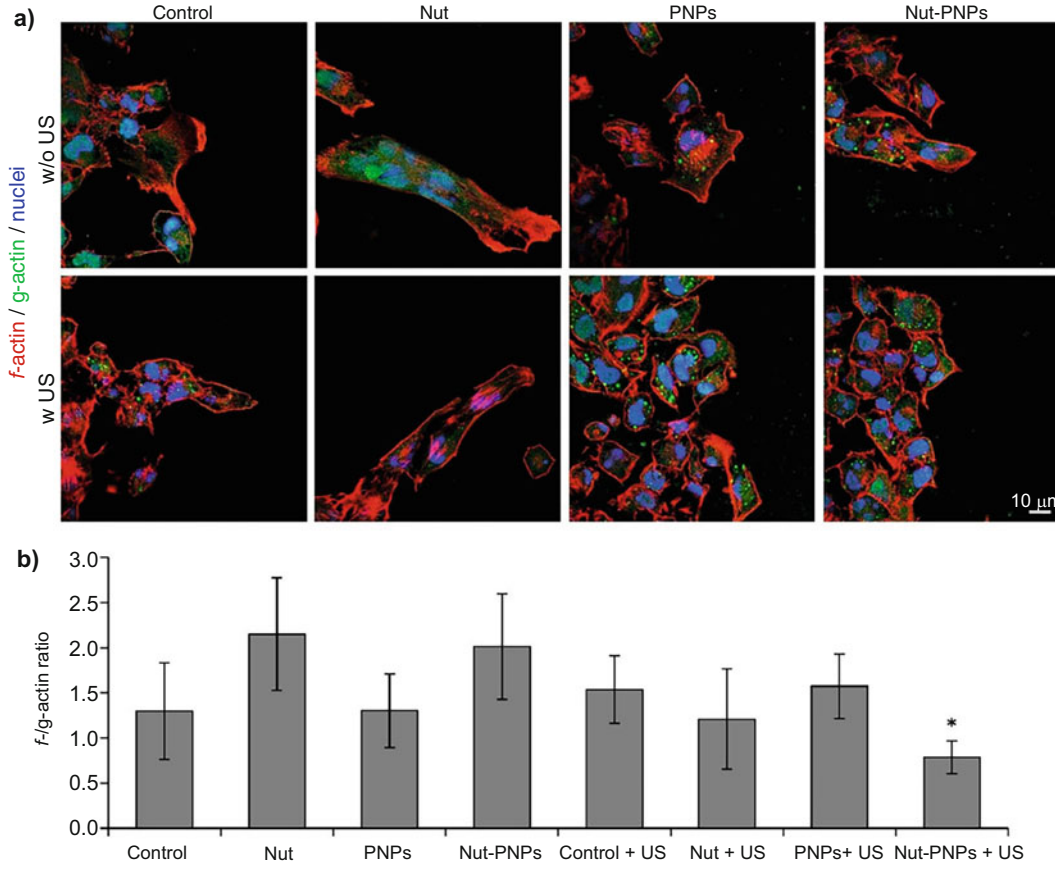

Fig. 2 (**a**) Representative confocal images of T98G cells at the scratch area (*g*-actin in green, *f*-actin in red, nuclei in blue); (**b**) quantification of the *f*-/*g*-actin ratio during cell migration ($* p < 0.05$). (Reproduced from [9] with permission from Elsevier)

8. After the incubation time, discard the media from the top chambers without altering the filter and the basement membrane matrix.
9. Remove the cells that did not undergo invasion from the top chamber by wiping with a cotton swab.
10. Disassemble the top chambers and set them aside.
11. Centrifuge the plate at 600 *g* for 5 min.
12. Discard the media from the bottom chambers, and rinse the cells with 500 μL of washing buffer.
13. Prepare 50 μL of cell dye (1:250 dilution in PBS with Ca^{2+} and Mg^{2+}) and mix 450 μL of cell dissociation buffer (quantity for a single well).
14. Add 500 μL of the prepared dye solution to each bottom well.
15. Place the top chambers into the bottom chambers, and incubate the plate for 30 min at 37 °C (*see* **Note 13**).

16. After incubation, remove the top chambers and transfer 100 μL of the solution to a 96-well plate.
17. Read the fluorescence (λ_{ex} 485 nm, λ_{em} 530 nm) with a fluorescence plate reader (VICTOR X3, PerkinElmer).
18. Convert the fluorescence in cell number using a standard curve.
19. Standard curve:
    (a) Dilute the cells serially in wash buffer (1:1, v/v) to generate a standard curve of T98G cells (cell number = 12500, 6250, 3125, 1562, 781, 390, 195, 98, and 49) in 100 μL total volume.
    (b) Use 100 μL wash buffer as blank.
    (c) To read the fluorescence of cells, follow the same procedure already described (*see* **Note 14**).

4 Notes

1. Particles were synthesized and surface-functionalized as previously described in a work of our group [9].
2. You may filter the prepared media prior to use.
3. We filtered the solution through a 0.45-μm membrane filter to remove any particles. PFA solution should be prepared freshly before use or can be stored in aliquots at −20 °C for several months. Avoid repeated freeze/thawing.
4. You may use ultraviolet light to sterilize surfaces in the cell culture hood.
5. Gently add PBS solution to the bottom part of the flask to avoid damaging the cell layer.
6. Note that the incubation time can vary depending on the cell line used.
7. You may gently tap the flask to favor cell detachment.
8. Note that the centrifugation speed and time can vary depending on the cell line used.
9. Avoid shaking the inserts. This might result into inhomogeneous cell distribution.
10. Check the confluency under a microscope. In case the cells did not reach 100% confluency, incubate them for a further 12–24 h checking the confluency regularly.
11. You may incubate the plate overnight at 4 °C.
12. Ensure that no air bubbles are trapped between the top and the lower chambers.

13. You may gently tap the plate on the side to ensure the dissociation.
14. Use triplicates for the standard curve.

5 Summary

In this chapter, we described three straightforward methodologies that can be performed when analyzing the effects of piezoelectric stimulation on cells' cytoskeleton and on their migration and invasion abilities. These assays were optimized for tumor cells, but they can be easily repurposed to other cell types; for instance, due to the scientific relevance, we envisage that these tests could be of extreme importance when evaluating the effects of piezoelectric stimulation on immune cells.

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Chapter 7

Preparation Method and In Vitro Characterization of Nanoparticles Sensitive to Tumor Microenvironment

Naym Blal and Daniela Guarnieri

Abstract

Immunotherapy is considered a powerful clinical strategy aiming to boost the immune system to fight cancer. In this context, nanomaterials (NMs) are uniquely suited to improve the development and the broad implementation of cancer immunotherapies by overcoming several challenges. In fact, NMs can be rationally designed to navigate complex physical barriers, respond to tumor microenvironments, and enhance/modulate immune system activation. Here, we present a method to prepare stimuli-responsive biocompatible nanoparticles (NPs) able to target the tumor microenvironment. Moreover, we describe protocols to characterize the physical–chemical properties of NPs as well as to evaluate their biocompatibility and therapeutic potential in vitro on three-dimensional (3D) tumor spheroids.

Key words Biocompatible nanoparticles, Nanoprecipitation, MMP-sensitive peptides, Tumor microenvironment, Drug delivery, Stimuli-responsive nanoparticles

1 Introduction

Due to their biological properties, solid tumors pose several challenges in the development of safe and effective treatments [1]. In fact, they usually have a highly fibrous matrix and abnormal blood flow that make it difficult for drugs to accumulate into target tissues [2, 3]. For instance, intravenously administered drugs accumulate in both healthy and diseased tissues and can be potentially picked up by the reticuloendothelial system (RES) [4], thus exhibiting a low therapeutic index and an increase in undesirable effects [5].

To address this problem, an alternative to chemotherapy is cancer immunotherapy. Cancer immunotherapy includes different approaches: checkpoint inhibitors, lymphocyte-promoting cytokines, engineered T cells, agonistic antibodies against co-stimulatory receptors, and cancer vaccines [6]. The common aim of these immunotherapy approaches is to enhance and improve the activation of the immune system towards the tumor, with fewer

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effects on healthy tissue than other chemotherapy drugs that directly kill cancer cells [7, 8].

Despite these advantages, immunotherapy also faces a series of critical issues of different nature. For example, this therapy is not effective for all patients, making it difficult to predict response [9]. In addition, it can cause autoimmune reactions in some patients that inevitably damage healthy tissue [10]. The search for new approaches to administer immunotherapy drugs in a safer and more controlled way could extend the efficacy of this therapy to an increased number of patients. Specifically, the development of new delivery technologies would allow targeted drug delivery into the tumor by reducing off-target effects.

Immunotherapy drug delivery platforms primarily investigated in recent years include scaffolds, implants, cell-based platforms, biomaterials, and nanoparticles (NPs) [11]. In particular, NPs are highly recommendable in cancer therapy of solid tumors thanks to their nanometric dimensions that – coupled to the polyethylene glycol (PEG) chains—allow to bypass RES and release the drug preferentially in the target tissues [12]. Furthermore, to increase the efficacy of drug delivery, drug-loaded NPs can be designed so that they release the drug following specific stimuli coming from the tumor microenvironment [13]. There are a lot of stimuli associated to tumor biochemical properties that can activate the target release of drug such as pH [14], reduction conditions [15], and overexpressed matrix metalloproteinases (MMPs) [16].

In this chapter, we describe a method to prepare a stimuli-responsive nanoparticle-based drug delivery system and the protocols to characterize its physical–chemical properties and to evaluate its therapeutic efficacy in vitro. To this aim, we use the nanoprecipitation method to form biodegradable NPs, based on poly(d, l-lactic-co-glycolic acid) (PLGA)–block–PEG copolymer (namely, PELGA). This method is very easy and versatile and allows to obtain NPs presenting specific features by combining different copolymers and conjugates [17, 18]. As an example of applicability of this method, here, the PELGA copolymer is blended with a tumor-activated prodrug (TAP) conjugated to PLGA. TAP is made of an MMP2 cleavable domain bound to doxorubicin (Dox), a well-known anticancer drug [17]. Techniques and procedures to determine the size, surface charge, colloidal stability, and drug release kinetics are reported in this chapter. Moreover, the assessment of biocompatibility as well as therapeutic efficacy of this nanosized drug delivery system on human glioblastoma (U87) and human dermal fibroblast (HDF) spheroids as a three-dimensional model of tumor and healthy tissues, respectively, are described in detail. The results show how to study the capability of these NPs to perceive the differences in the levels of endogenous MMP2

enzymes and to modulate the drug release inside tumor spheroids, accordingly. Furthermore, this chapter highlights the potentiality of employment of NM-based systems to implement and improve cancer immunotherapy strategies.

2 Materials

2.1 Copolymers and Conjugates

To prepare NPs used in this work, the following reagents are employed:

1. PELGA copolymer, synthetized via a coupling reaction between poly(d,l-lactide-co-glycolide) (PLGA) (Resomer RG502H, Mw 12,000 Da, Boehringer, Germany) and polyethylene glycol (PEG) (Mw 1500 Da, Sigma–Aldrich, USA), according to a protocol described elsewhere [19];
2. Doxorubicin (Dox) (doxorubicin hydrochloride, purity >99%, Discovery Fine Chemicals, UK) (*see* **Note 1**).
3. MMP2-sensitive peptide (Fmoc-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Gln-COOH), synthetized with the standard solid-phase Fmoc method [20] (*see* **Note 2**). Amino acids for MMP2 peptide synthesis are purchased from IRIS Biotech GmbH, Germany.
4. Dox is covalently conjugated to PLGA chains to form a PLGA–Dox conjugate or to an MMP2-sensitive peptide to form the tumor-activated prodrug (TAP) and, consequently, to PLGA to form PLGA–TAP according to procedures previously described [17].
5. The copolymers and conjugates are combined to prepare PELGA–Dox and PELGA–TAP nanoparticles as reported in Fig. 1.

2.2 Cell Culture Reagents

1. To test cell response to NPs, human glioma cell line (U87, ATCC) and human dermal fibroblasts (HDF, ECACC) are used as a model of tumor and healthy tissues, respectively (*see* **Note 3**).
2. U87 are grown in complete Eagle's minimal essential medium (EMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin (Euroclone).
3. HDFs are grown in EMEM supplemented with 20% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and nonessential amino acids (Euroclone).
4. Cells are incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

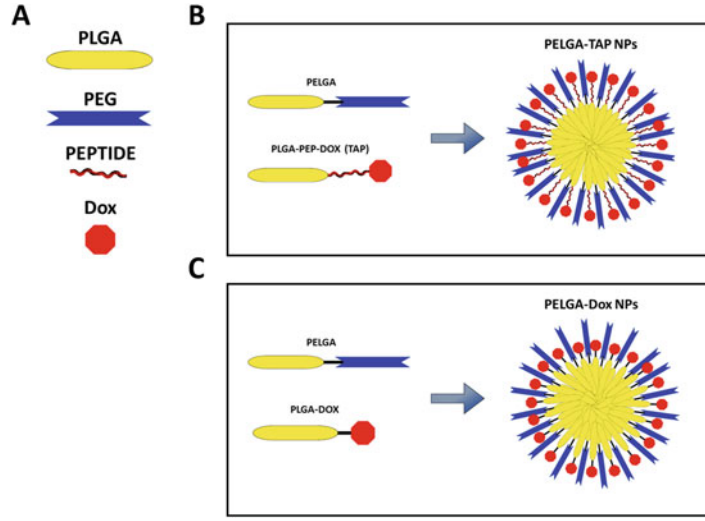

Fig. 1 Schematic representation of each single component (namely, PLGA, PEG, MMP2-sensitive peptide [peptide], and doxorubicin [dox]) and their combination to synthetize different copolymers used as building blocks to prepare nanoparticles (**a**). MMP2-sensitive PELGA–TAP NPs (**b**) and PELGA–Dox NPs (**c**)

2.3 MMP2 Cleavage Assay

1. Dissolve 3.9 mg of p-aminophenylmercuric acetate (APMA) (Sigma-Aldrich, USA) in 1 mL of 0.1 M NaOH to obtain a 100 μM APMA solution.
2. Dilute this solution with 99 mL of 50 mM Tris–HCl buffer (pH 7.5) (Sigma-Aldrich, USA) and adjust to pH 7.2.
3. A 20 nM MMP2 solution is prepared dissolving recombinant human matrix metalloproteinase 2 (MMP2) (purchased from Peprotech Inc., USA) in MMP2 buffer solution (50 mM HEPES, 200 mM NaCl, 10 mM $CaCl_2$, 1 mM $ZnCl_2$, pH 7.4) (Sigma-Aldrich, USA).
4. Dialysis bags (MWCO 6000–8000 Da) are purchased from Spectrum Laboratories, Inc. (the Netherlands).

2.4 Nanoparticle Cytotoxicity

The biocompatibility of PELGA NPs is evaluated through different assays by using rhodaminated PELGA NPs prepared by combining PELGA copolymer and rhodamine-conjugated PLGA (Rhodamine B, Sigma-Aldrich, USA).

2.4.1 Alamar Blue Assay

1. Perform this assay on 70–80% confluent cells of U87 and HDF cell culture.
2. Dilute Alamar Blue reagent (Invitrogen) in DMEM w/o phenol red at 10% final concentration (v/v).
3. Measure the absorbance by a plate reader (Wallac Victor 1420 Victor2™, Perkinelemer, USA).

2.4.2 Confocal Microscope Imaging

1. Dissolve paraformaldehyde powder in phosphate-buffered saline (PBS) (Sigma-Aldrich, USA) at about 100 °C under stirring to obtain 4% paraformaldehyde solution. Ensure the solution becomes transparent, then make aliquots of 4–5 mL in 15-mL conic tubes, and store them at −20 °C (*see* **Note 4**).
2. Dilute Triton X-100 (Sigma-Aldrich, USA) in PBS under stirring to obtain 0.1% Triton X-100 (*see* **Note 5**).
3. Localize cell nuclei by incubating samples with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich, USA) diluted 1:10000 in PBS.
4. Proceed to observation with a confocal microscope (Leica TCS SP5 MP).

2.4.3 Quantification of NP Cellular Uptake Kinetics

1. Dilute Triton X-100 stock solution in PBS to get a 1% Triton X-100 lysis buffer.
2. Quantify the fluorescence by spectrofluorometer (Wallac 1420 Victor2™, PerkinElmer, USA).

3 Methods

3.1 NP Preparation

1. To prepare PELGA–TAP NPs, dissolve 1 mg of PELGA and 1 mg of PLGA–TAP in 500 μL of acetone and mix the solution (*see* **Note 6**).
2. Move the solution into a 10-mL syringe and mount the syringe on a syringe pump (Harvard Apparatus).
3. Add the solution dropwise (6 mL/h) (*see* **Note 7**) into a beaker containing 12.5 mL of distilled water (18 MΩ resistance purchased from Millipore, USA) under magnetic stirring (600 rpm) using the syringe pump (Fig. 2).
4. Allow the organic solvent to evaporate for 3 h to obtain a NP dispersion (*see* **Note 8**).
5. Sterilize the NP dispersion by filtering with 0.22-μm membrane filter (*see* **Note 9**).
6. Decrease to 1 mL the volume of the NP suspension with Amicon Ultra4 10-kDa centrifuge tube (Millipore) to obtain a final NP concentration of 2 mg/mL (*see* **Note 10**).
7. Prepare PELGA–Dox NPs (without the MMP2-sentive peptide), used as a negative control, with the same protocol described at points 1–6 starting by dissolving 1 mg of PLGA–Dox and 1 mg of PELGA in 500 μL of acetone.
8. Prepare rhodaminated PELGA (PELGA–rhod) NPs according to the above procedure for preliminary biological characterization of NPs.

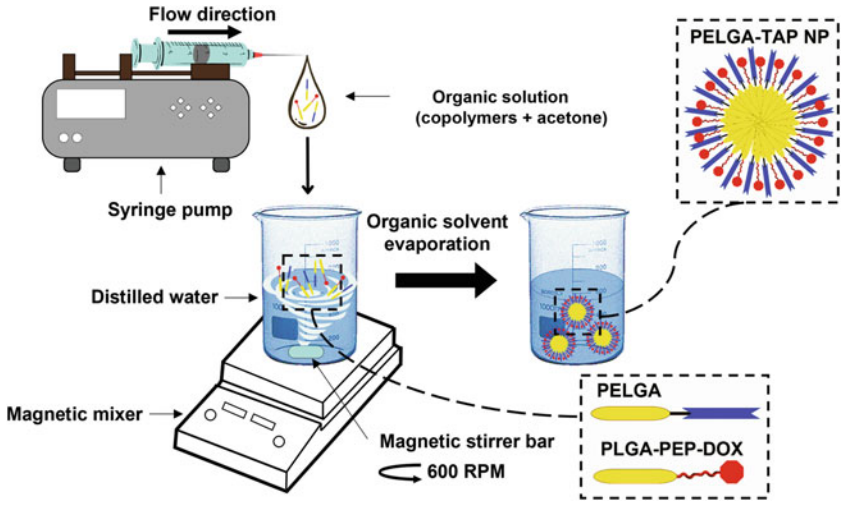

Fig. 2 Scheme of NP preparation according to the nanoprecipitation method

Table 1
Size and ζ-potential of PELGA–TAP and PELGA–Dox NPs (polydispersity index [PDI] < 0.2). Dox/NP ratios are 20 g/mg for PELGA–TAP and 21.5 g/mg for PELGA–Dox. Data are reported as mean ± SD. $p < 0.05$ is considered statistically significant

Sample	Mean size (nm±SD)	ζ-potential (mV±SD)
PELGA-TAP	75.1 ± 0.5	−29.5 ± 14.8
PELGA-Dox	62.0 ± 0.9	−23.6 ± 12.8

3.2 PELGA NP Physical–Chemical Characterization

The NPs, obtained according to the procedures described above, are characterized by measuring the mean size, size distribution, and ζ-potential by dynamic light scattering (DLS). NP morphology is analyzed by scanning electron microscopy (SEM) and cryogenic transmission electron microscopy (Cryo-TEM).

3.2.1 DLS

1. Measure NP mean size, size distribution, and ζ-potential by ZetaSizer Nano ZS (Malvern Instruments, UK). Mean size and ζ-potential are reported in Table 1.
2. Perform the analysis on a 0.1 mg/mL NP suspension in water (*see* **Note 11**).
3. To evaluate NP stability over time, repeat the DLS measurements after 1, 7, and 14 days after NP preparation.
4. Store NPs at 4 °C between one measure and another.

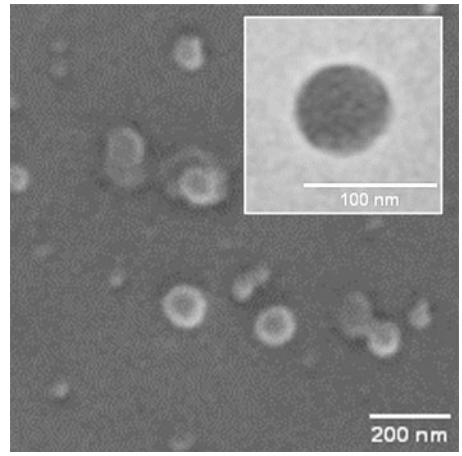

Fig. 3 SEM micrograph of PELGA-TAP NPs. The inset shows Cryo-TEM micrograph of PELGA–Dox NPs at high magnification

3.2.2 SEM

1. Deposit 50 μL of NP suspension on a coverslip mounted on a standard SEM pin stub.
2. Gold-sputtering (3-nm thickness) the samples with a HR208Cressington sputter coater.
3. Analyze the samples with FESEM ULTRAPLUS (Zeiss) at 20 kV with the SE2 detector and 15.9-mm working distance (Fig. 3).

3.2.3 Cryo-TEM

1. Vitrify the samples with FEI Vitrobot Mk IV (*see* **Note 12**).
2. Place 3 μL of sample on 200 mesh Quantifoil grids.
3. Blot the excess sample with filter paper (*see* **Note 13**).
4. Immerse the grid into liquid propane (*see* **Note 14**).
5. Perform imaging by TEM TECNAI G^2 equipment at 200 kV in low-dose mode and Eagle 2HS camera (Fig. 3).

3.3 Biocompatibility of PELGA NPs

After characterizing the NPs from a physicochemical point of view, we proceed with the evaluation of their biocompatibility to exclude the toxicity due to any residues of solvents or other reagents used during the NP preparation. For this purpose, fluorescent NPs obtained by conjugating PLGA to rhodamine are used. PELGA–rhod are tested on U87 and HDF cells in two-dimensional (2D) standard cell culture at the final concentration of 0.05 mg/mL in cell culture medium for specific timepoints. NP cytotoxicity is tested by Alamar Blue assay, while NP cellular uptake is evaluated by confocal microscopy and spectrofluorimetric analysis.

3.3.1 Alamar Blue Assay

1. Seed 1×10^4 cells/well in a 96-well microplate at a final volume of 100 μL culture medium.
2. Incubate the microplate for 24 h at 37 °C and 5% CO_2.

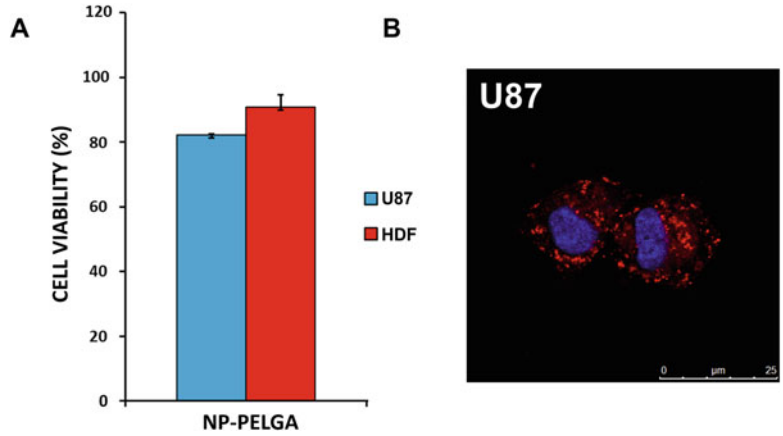

Fig. 4 Cell viability percentage of U87 (blue) and HDF (red) cell lines incubated with PELGA NPs for 24 h determined by Alamar Blue assay (**a**). Data are reported as mean ± standard deviation (SD) of viable cell percentage normalized to non-treated cells. Representative image of intracellular distribution of PELGA–rhod NPs (red) in U87 cells; DAPI staining shows cell nuclei (blue) (**b**)

3. To assess NP cytotoxicity, incubate cells for 24 h at 37 °C with culture medium containing 50 μg/mL PELGA–rhod NPs at a final volume of 100 μL.
4. After incubation, remove the media and wash the cells with 100 μL PBS.
5. Add 100 μL of culture medium w/o phenol red containing 10% Alamar Blue reagent.
6. Incubate the cells for 4 h at 37 °C.
7. Measure the absorbance at 570 and 600 nm.
8. Report data as percentage of viable cells normalized to non-treated cells (Fig. 4a).

3.3.2 Confocal Microscopy

1. Seed 5×10^4 cells on a 12-mm glass coverslip in a 24-well plate with in 1 mL of medium.
2. After 24 h from cell seeding, change the medium with 1 mL of fresh medium containing 50 μg/mL PELGA–rhod NPs.
3. Incubate for 24 h at 37 °C.
4. At the end of incubation, remove the medium and rinse the cells twice with PBS to remove non-internalized NPs.
5. Fix the cells with 4% paraformaldehyde for 20 min at room temperature.
6. Stain the cells nuclei with DAPI for 10 min at room temperature.
7. Rinse twice with PBS and once with ddH_2O.

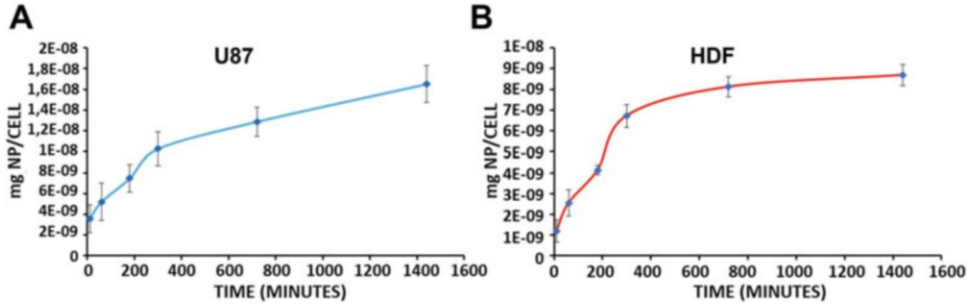

Fig. 5 Uptake kinetics of PELGA–rhod NPs in U87 (**a**) and HDF (**b**) cells. Data are reported as mean ± SD

8. Mount the glass coverslip on a glass slide.
9. Observe at confocal microscope with a 63× oil-immersion objective.
10. Acquire images at confocal microscope with a resolution of 1024 × 1024 pixels (Fig. 4b).

3.3.3 Uptake Quantification Analysis

1. Seed 5×10^4 cells in a 24-well plate with in 1 mL of medium.
2. After 24 h from cell seeding, change the medium with 1 mL of fresh medium containing 50 μg/mL PELGA–rhod NPs.
3. Incubate for different timepoints up to 24 h at 37 °C.
4. At each timepoint, remove the medium and rinse the cells twice with PBS.
5. Lyse the cells with 1% Triton X-100 in PBS.
6. Measure the fluorescence of the cell lysates with a spectrofluorometer at $\lambda_{ex} = 543$ nm.
7. Quantify the internalized NPs by interpolating the fluorescence intensity data with a calibration curve of PELGA–rhod NPs (Fig. 5).

3.4 MMP2-Mediated Drug Release Assay

Dox release is evaluated in vitro by MMP2 cleavage assay, performed in MMP2 buffer solution as previously reported [20].

1. To allow activation of MMP2 enzyme, add 1.8 μL of 100 μM APMA solution to 2 mL of 20 nM MMP2 and incubate for 3 h at 37 °C.
2. After MMP2 enzyme activation, fill a 50-mL centrifuge tube with 12.5 mL of the MMP2 buffer solution prepared as described at **item 3** of Subheading 2.3.
3. Add 500 μL of PELGA–TAP or PELGA–Dox suspensions to a dialysis tube (MWCO 6000–8000 Da) in the presence or absence of 20 nM activated MMP2 enzyme.
4. Immerge the dialysis tube in the centrifuge tube containing MMP2 buffer and incubate at 37 °C under stirring (100 rpm).

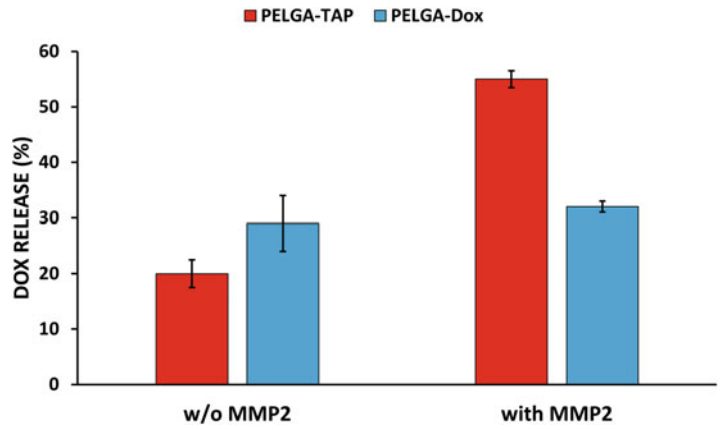

Fig. 6 Percentage of Dox release after 48 h of incubation at 37 °C, in the presence or absence of the MMP2 enzyme, from PELGA–TAP and PELGA–Dox NPs. Data are reported as mean ± SD

5. Collect 120 μL of released medium at different timepoints and quantify the released Dox by spectrofluorometric measurements ($\lambda_{ex} = 485$ nm; $\lambda_{em} = 595$ nm) (*see* **Note 15**).
6. Calculate the percentage of released Dox considering as 100% the amount of free Dox diffused through the dialysis tube (*see* **Note 16**) as shown in Fig. 6.

3.5 Spheroid Formation

1. For spheroid formation, detach U87 and HDF cells at about 70–80% of confluency with 1 mL trypsin/EDTA solution at 37 °C for 5 min.
2. Harvest the cells in a 15-mL tube with 9 mL fresh cell culture medium, centrifuge at 1000 rpm, discard the supernatant, and resuspend the pellet in fresh cell culture medium.
3. Count the cells.
4. Seed about 2500 U87 cells and about 3000 HDF cells in each well of a nonadherent round-bottomed 96-well plate (Greiner, Frickenhausen, Germany) containing 0.25% (w/v) carboxymethylcellulose in cell culture medium.
5. Incubate the sample at 37 °C, 5% CO_2 and 100% relative humidity for 3 days to allow spheroid formation.
6. Check quality of spheroids by optical microscope (*see* **Note 17**) (Fig. 7, left panels).

3.6 Cytotoxicity Test

1. Harvest the spheroids and seed them in low-attachment 6-well plates to have about 6–10 spheroids in each well.
2. After 24 h, add 1 mL of cell culture media containing PELGA–Dox NPs or PELGA–TAP NPs and incubate at 37 °C.

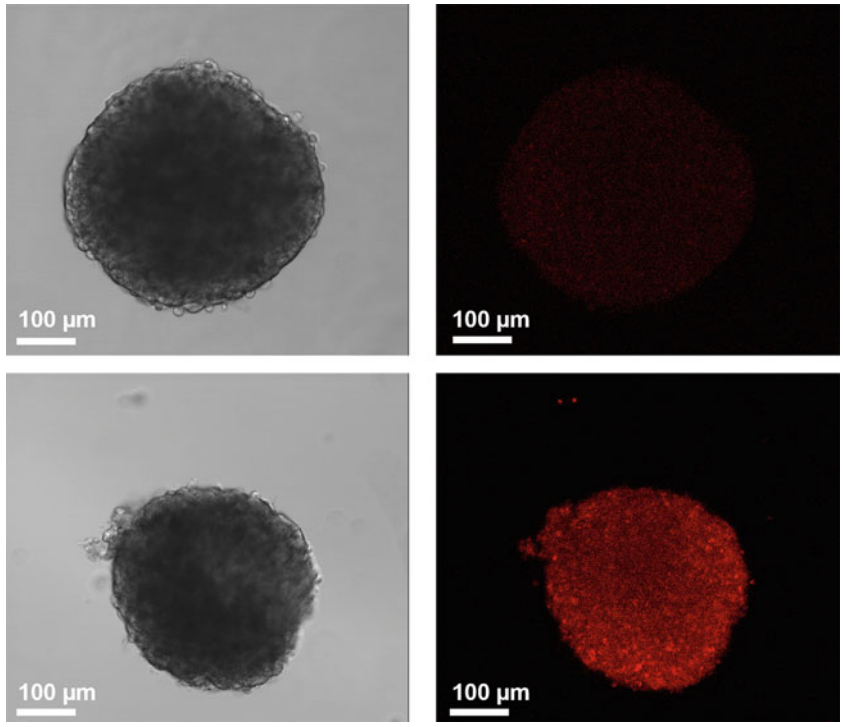

Fig. 7 Representative fluorescence and transmitted light images of U87 spheroids after 48-h treatment with PELGA–Dox and PELGA–TAP NPs. Red fluorescence indicates Dox accumulation within spheroids

3. After 24 and 48 h, remove cell culture medium and wash twice with PBS (*see* **Note 18**).
4. Fix the spheroids with 4% paraformaldehyde for 30 min at room temperature.
5. Wash twice with PBS (*see* **Note 19**).
6. Observe the fluorescence of Dox diffused into the spheroid matrix by confocal microscope (Leica) with a 10× objective (Fig. 7).
7. Acquire z-sectioning images with a resolution of 1024 × 1024 and 488-nm laser.
8. Measure the fluorescence intensity (Fig. 8) and mean spheroid diameter (Fig. 9) with confocal microscope software (*see* **Note 20**).
9. For fluorescence intensity, report the data as the distribution of normalized pixel counts as a function of grey scale value of each pixel ranging from 0 to 255.

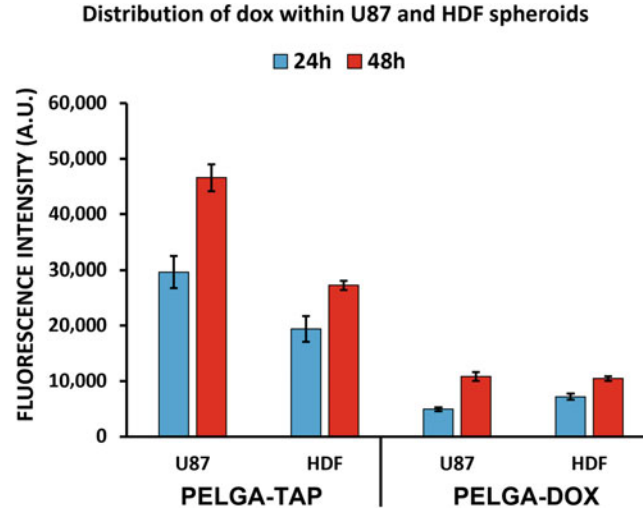

Fig. 8 Fluorescent intensity distribution of Dox within U87 and HDF spheroid after 24 and 48 h. The *y* axis represents the fluorescent intensity of spheroid expressed as mean grey values. Data are reported as mean ± SD

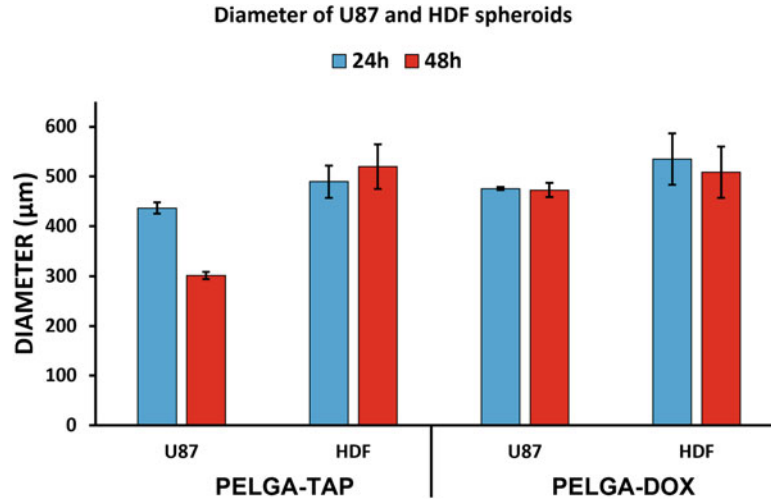

Fig. 9 Diameter of U87 and HDF spheroids after 24- and 48-h treatment with PELGA–TAP or PELGA–Dox. Data are reported as mean ± SD

4 Notes

1. Handle with care; it can cause genetic alterations and cancer. Work under fume hood. Do not inhale the substance/mixture. Wear gloves, protective clothing, eye protection, and protect the face. Wash skin carefully after use.

2. As an alternative to synthesis, these polymers can be customized and purchased from specialized companies.
3. Cell lines were selected according to their different expression levels of MMP2 enzyme evaluated through indirect immunofluorescence and zymography [17]. In particular, in our experimental conditions, U87 cells express MMP2 enzyme levels eight-fold higher than HDF cells [17].
4. Thaw 4% paraformaldehyde in a thermostatic bath at 37 °C prior to use and maintain it at 4 °C. Do not refreeze.
5. Store the solution at room temperature.
6. Make sure there are no precipitates and wait until the solution becomes clear.
7. Ensure that the drop falls within the water whirl and not sideways. Pay attention to the velocity because it can change the mean size and size distribution of the nanoparticles.
8. Wait more than 3 h to be sure that the acetone is completely evaporated, because acetone residues can be toxic.
9. From this step it is necessary to work in sterile conditions.
10. After NP preparation proceed with size measurements as described in Subheading 3.2 and store NPs at 4 °C to avoid peptide degradation.
11. Average the results on at least five measurements.
12. Make sure that you are in a saturated water vapor environment during this operation.
13. Blot and drying time are both 1 s.
14. Liquid propane needs to be cooled by liquid nitrogen that is surrounded by the propane vessel.
15. For this assay, the intrinsic fluorescent of Dox is exploited. It is worth to note that for Dox concentrations higher than 1 mg/mL, a self-quenching phenomenon can occur thus leading to misinterpretation of experimental data. To avoid such issue, perform fluorescence measurements of the sample at its original concentration and after dilution to check the decrement in fluorescence intensity.
16. In order to quantify the Dox maximum release, a dialysis bag (MWCO 10000 Da) is loaded with 500 μL of free doxorubicin (40 μg/mL) and incubated in 12.5 mL of MMP2 buffer at 37 °C under stirring (100 rpm). At scheduled time intervals, 120 μL of the buffer is analyzed using a spectrofluorometer, until reaching the plateau.

17. Check the following parameters before use spheroids for experiments: round and symmetric shape; regular and well-defined boundaries; size around 200 μm.
18. Do not remove the cell culture medium completely in order to avoid aspirating spheroids with the pipette accidentally. It is better to leave a few microliters of medium in the well and, in case, to increase the washing steps with PBS.
19. Keep spheroids in PBS. Do not dry the samples. They can be stored at 4 °C.
20. Fluorescence intensity can be measured also by using other image analysis software such as ImageJ.

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Chapter 8

A New Microfluidic Device to Facilitate Functional Precision Medicine Assays

Albert Manzano-Muñoz, Jose Yeste, María A. Ortega, Josep Samitier, Javier Ramón-Azcón, and Joan Montero

Abstract

Functional precision medicine (FPM) has emerged as a new approach to improve cancer treatment. Despite its potential, FPM assays present important limitations such as the number of cells and trained personnel required. To overcome these impediments, here we describe a novel microfluidic platform that can be used to perform FPM assays, optimizing the use of primary cancer cells and simplifying the process by using microfluidics to automatize the process.

Key words Functional assays, Precision medicine, Microfluidics, Cancer treatment

1 Introduction

Most initiatives aiming to personalize medicine to treat cancer patients are focused on molecular analyses to find targetable alterations [1]. In this regard, functional assays represent a great opportunity to improve cancer treatment, since they can help identify the best treatment for each patient. Several examples have been successfully assessed preclinically, but a common major limitation is the number of cells needed to perform these assays [2]. To overcome this impediment, some functional technologies expand the biological material obtained from patients, for example, through organoids or patient-derived xenografts that have obtained good predictive results [3, 4]. However, these techniques require extensive time and resources, which limits their clinical implementation. Alternatively, we can directly expose patient-isolated cancer cells to cytotoxic agents to determine their efficacy. For instance, techniques such as pharmacoscopy, dynamic BH3 profiling, or single-cell

Javier Ramón-Azcón and Joan Montero contributed equally with all other contributors.

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mass measurements have obtained excellent results, also in the clinic [5–9]. When analyzing liquid tumors, obtaining enough cells to perform ex vivo functional assays is very feasible, but it is more complicated for solid tumor biopsies, where the number of viable cells obtained normally does not allow a large screening of molecules. Yet microfluidic platforms could be used to decrease these requirements, maximizing the treatments that could be tested and increasing the likelihood of finding an effective therapy.

Here we present a novel microfluidic platform that exposes (primary) cancer cells to a lineal increasing concentration of a given cytotoxic agent, allowing its assessment using fluorescent probes [10]. This microfluidic design requires minimal handling and just a basic equipment (for example, a peristaltic pump and a fluorescence microscope), avoiding the requisite of trained personnel and complicated procedures that will facilitate the implementation of functional assays in hospitals to benefit cancer patients.

This microfluidic chip can be easily scaled up and, because of its reduced cell number requirement, this platform can help introduce functional assays as a normal procedure to assign the best possible treatment for cancer patients.

2 Materials

2.1 SU8 Mold Fabrication

1. 4″ n-type <100> silicon wafer (MicroChemicals).
2. Oxygen and Air Plasma cleaner (Harrick Plasma – model PCD-002-CE) operating at three modes of power: 7.2 W (low), 10.2 W (medium), and 29.6 W (high).
3. Negative SU-82100 photoresist (MicroChem Lab).
4. Spin-coater machine (Laurell Tech – model WS-650MZ 23NPP/LITE).
5. UV-photolithography mask aligner (SÜSS Microtec model MJB4).
6. Photomask: microfluidic design in high-resolution transparent film (JD Photodata UK).
7. SU-8 developer (MicroChem Lab).
8. 2-Propanol.
9. Tricholoro perfluoroctyl silane (TPS) (Sigma-Aldrich, 448931) to perform silanization of the molds.

2.2 PDMS Replica Molding

1. Sylgard 184 (prepolymer and curing agent).
2. 75 × 50 mm glass slide.
3. Desiccator connected to a vacuum line.
4. 0.5-mm multipurpose sampling tool puncher.

5. Oxygen and Air Plasma cleaner (Harrick Plasma – model PCD-002-CE).
6. 4-mm multipurpose sampling tool puncher.

2.3 Microfluidic-Based Dynamic BH3 Profiling

1. Coating solution: 15 μg/mL of poly-L-lysine (Quimigen) in sterile MilliQ water.
2. Complete media: For primary samples a standard cell culture media can be used, for example, RPMI with 10% FBS, 1% L-glutamine, and 1% pen-strep. In case of cells with different needs, use a specialized media.
3. Plastic alligator clips.
4. Microfluidic pressure pump.
5. Staining solution: complete media used to culture the cells with the desired concentration of fluorescent dyes. Staining might include nuclear dyes (DAPI 1 μM, Thermo Fisher), viability dies (calcein AM 4 μM, Thermo Fisher), apoptotic initiation dyes (TMRE 400 nM, Abcam), or death identification dyes (propidium iodide 1 μM, Thermo Fisher).
6. Phosphate-buffered saline (PBS).
7. Cytotoxic induction solution: complete media with cytotoxic agent. Depending on the type of tumor studied, different chemotherapies or targeted agents can be used, for example, doxorubicin 100 nM (SelleckChem) or trametinib 100 nM (SelleckChem).

3 Methods

3.1 SU8 Mold Fabrication to Create PDMS Microfluidic Chip

1. Design the pattern of the photomask using a computer-aided design (CAD) software and print it (*see* **Note 1**). This will include, as nontransparent regions, chambers for plating the cells and microfluidic channels for splitting the solution and generating the concentration gradient (Fig. 1).
2. Put the silicon wafer in an oxygen plasma cleaner at low power mode for 20 min (*see* **Note 2**).
3. Transfer the silicon wafer to a hot plate and bake at 95 °C for 5 min.
4. Place the silicon wafer on the spin coater and dispense enough SU-82100 photoresist over the wafer to cover all the surface. Apply in the spin coater 500 rpm with an acceleration of 100 rpm/s for 5 s followed by 3000 rpm with an acceleration of 300 rpm/s for 30 s to obtain a homogenous layer of 100 μm.

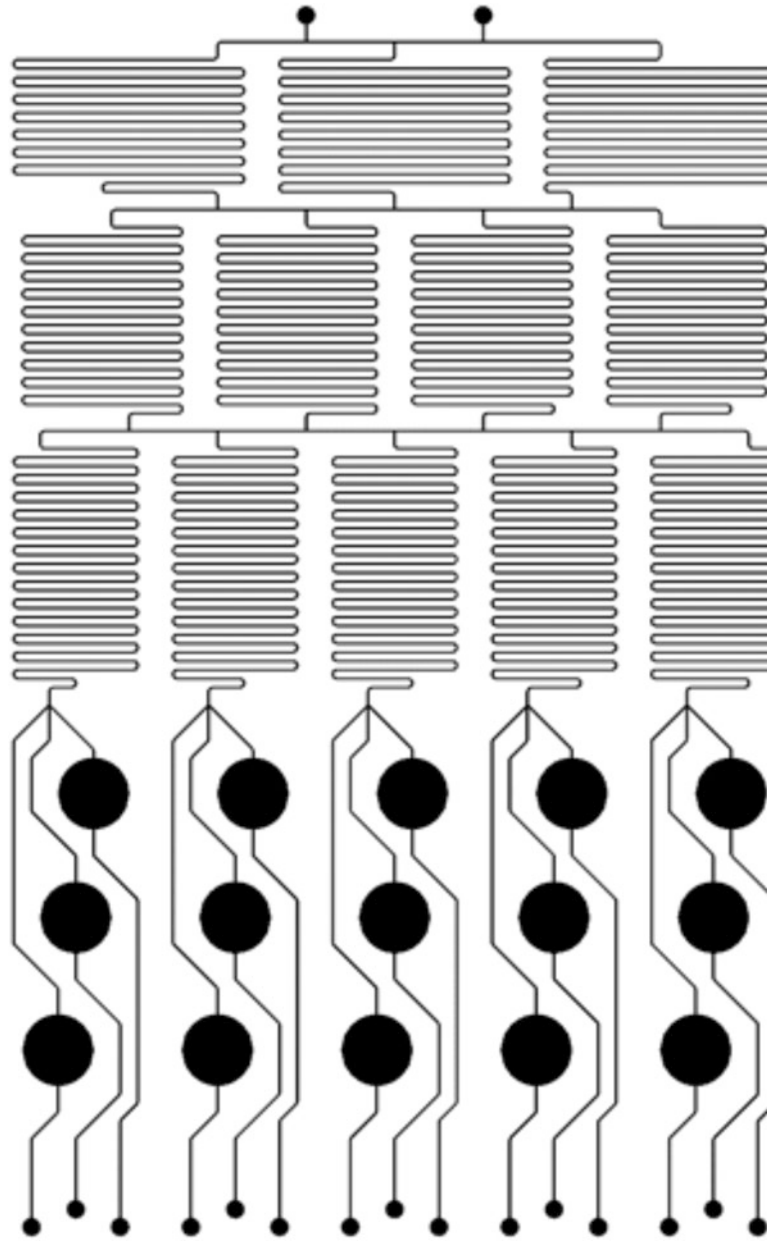

Fig. 1 Design of the microfluidic platform for cancer functional assays

5. Evaporate all solvents by baking at 65 °C for 5 min followed by 95 °C for 20 min.
6. Bring the silicon wafer to a mask aligner and, with the photomask on top, expose the SU-8 photoresist with UV light at 240 mJ/cm^2 of energy. Crosslink the photoresist by baking the silicon wafer on a hot plate at 65 °C for 5 min followed by 95 °C for 10 min.

7. Remove the labile photoresist by immersing the silicon wafer in the SU-8 developer for 10 min and then wash with 2-propanol. Repeat immersion in SU-8 developer for 1 min and 2-propanol wash until microfluidic features are clearly patterned on the wafer. Inspection under an optical microscope using a UV filter.
8. Place the SU8 mold on a hot plate at 150 °C for 60 min. Then, turn off the hot plate and allow to cool down to room temperature.
9. Silanize the surface of the SU8 mold to produce a hydrophobic coating to prevent the PDMS from adhering to the mold. Place the silicon wafer inside a plastic desiccator, containing a reservoir with 3–4 drops of TPS. Leave it closed and apply vacuum for 30 min. This step must be performed in a fume hood.

3.2 PDMS Microfluidic Chip Production

1. Weigh the amount of Sylgard 184 prepolymer to generate a PDMS layer of the desired thickness (*see* **Note 3**). Do this procedure in separate containers for every layer of PDMS. Add a 1:10 ratio of Sylgard 184 curing agent and mix thoroughly for at least 3 min (*see* **Note 2**).
2. Pour the PDMS mix into three Petri dishes according to the size of the desired layer. In two separate empty Petri dishes, pour the amount of mix to form two 1-mm-thick layers. In the third Petri dish, containing the SU8 mold with the microfluidic design, pour the PDMS mix calculated to generate a 2-mm-thick layer.
3. Put the Petri dishes in a desiccator and apply vacuum for at least 30 min to ensure that all bubbles in the PDMS mix disappear (*see* **Note 4**).
4. Clean a 75 × 50 mm glass slide in a sonicator. Submerge the glass in a mix of water and soap and sonicate for 5 min. Repeat the same procedure with water, ethanol, and 2-propanol for 5 min each. Dry the slide with a N_2 gun.
5. In one of the Petri dishes with 1 mm of PDMS mix, press the glass slide against the uncured mix to obtain a thin layer over the glass (*see* **Note 5**).
6. Leave all Petri dishes overnight on a leveled surface at room temperature followed by 4 h in an oven at 85 °C (*see* **Note 6**).
7. Using a scalpel cut the cured PDMS and carefully peel off the three layers: (1) the 2-mm-thick layer with the microfluidic motifs, (2) the plain 1-mm PDMS layer, and (3) the glass slide coated with a thin layer of PDMS.
8. Using a 0.5-mm multipurpose puncher, pierce the inlets and outlets in the 2-mm PDMS layer (*see* **Note 7**).

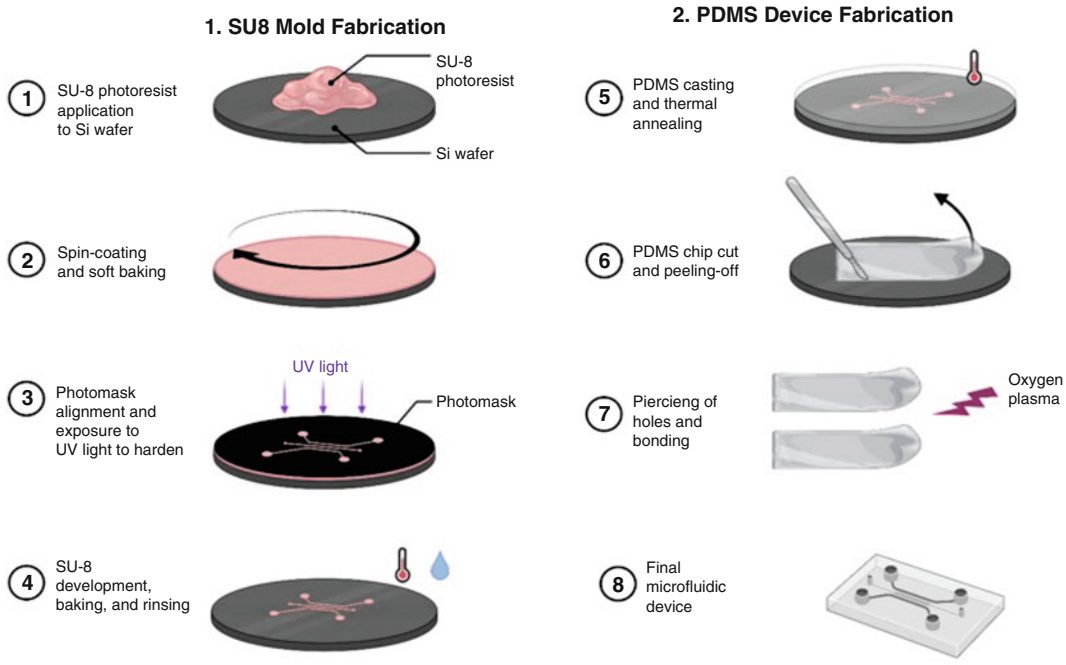

Fig. 2 Schematical summary of the microfluidic mold fabrication and the PDMS replica molding process to generate the microfluidic platform

9. Activate the 1-mm and 2-mm PDMS layers in an oxygen plasma cleaner at a constant oxygen pressure of 25 bar and 10.5 W for 30 s. Immediately, bring into contact both activated PDMS surfaces, gently press, and bake in an oven at 85 °C for 4 h to stabilize the covalent bonds.
10. Using a 4-mm multipurpose puncher, pierce the cell chambers in the resulting 3-mm-thick PDMS layer (*see* **Note 7**).
11. Activate the final 3-mm PDMS layer and the glass slide in the oxygen plasma cleaner using the same conditions as in 9. Press both parts and bake for 4 h at 85 °C. A summary of the production process is represented in Fig. 2 and the final platform shown in Fig. 3.

3.3 Functional Assays Using the Microfluidic Platform

1. Sterilize the microfluidic chip in an oven for 1 h at 85 °C (*see* **Note 8**).
2. Add 10 μL of coating solution to every cell chamber and incubate for 40 min at 37 °C. Then, wash the chambers with sterile MilliQ water (*see* **Note 9**).
3. Resuspend 30,000 of the desired cells in 200 μL of complete media to obtain the cell suspension to seed (*see* **Note 10**).

Fig. 3 Image of the final microfluidic platform

4. Seed 35 μL of the cell suspension in each well to obtain one experimental condition in every independent row of the microfluidic chip. Incubate at 37 °C inside an incubator enough time for the cells to attach (*see* **Note 11**).
5. If media is evaporated during the incubation, fill again the cell chambers with complete media and seal the microfluidic chip with a new glass and the alligator clips (*see* **Note 12**).
6. Connect with microfluidic tubing the pressure-base flow controller to the two inlets of the microfluidic chip (*see* **Note 13**). At this point, depending on the design of the experiment to generate the cytotoxic agent gradient, two solutions (one with the maximum cytotoxic agent concentration desired and the other with media) will be perfused through the two inlets (*see* **Note 14**). Cleanings with PBS or staining all the wells can be performed by perfusing the same solution through both inlets.
7. After cell incubation, to allow the effect of the cytotoxic induction solution, use a fluorescent microscope to take images in every cell chamber and quantify the desired biological parameter.

4 Notes

1. Multiple companies offer services for high-resolution printing on transparent films (for example, JD Photo Data, UK).

2. Both the SU8 mold fabrication and the PDMS replicates should be done in a clean room where the number of particles, humidity, and temperature are controlled.
3. To calculate the weight of the PMDS mix to obtain a specific thickness, the following formula was applied: $m = \pi r^2 h\rho$, where m is the mass of polymer mix in grams, r is the radius of the Petri dish in cm, h is the desired thickness in cm, and ρ is the density of PDMS (0.965 g/cm^3).
4. If air bubbles do not disappear from the PDMS uncured mix, applying air with a dropper or a N_2 gun at low pressure can help to pop the remaining bubbles.
5. To avoid deposition of PDMS in the opposite face of the glass slide, before pressing down to the uncured mix, the upper face can be covered with adhesive tape that can be easily eliminated after the curing process.
6. It is important to cure the PDMS on a flat surface to obtain even microfluidic chips. Ensure that overnight incubation is done on a flat surface using a level. Alternatively, if the oven is placed in a flat position, curing can be done by baking at 60 °C overnight.
7. To ensure a good piercing of the inlets and outlets, a magnifying camera can be used to correctly position the puncher. Holes must be made with the puncher in a straight position, perforating all the PDMS layers and rotating the puncher at the end to ensure a clean hole. It is important to check that the cut PDMS part is discarded to avoid inlet or outlet clogging.
8. Alternatively, the UV sterilization program of a cell culture hood can be used.
9. Surface functionalization can be applied depending on the cells, such as fibronectin, collagen, or laminin. Use the same protocol to functionalize culture surfaces with the desired molecule in the cell chambers of the microfluidic chip.
10. In this step, sequential cytotoxic agents can be added, for example, to identify a synergistic combination of two agents.
11. A small Petri dish full of MilliQ water can be placed next to the microfluidic chip to minimize evaporation of the cell culture media.
12. This is a critical point; if there are some big air bubbles when sealing the chip, the microfluidic circuit cannot be properly established. Moreover, if the chip is uneven, the sealing might fail, and some chambers might leak. To prevent it, place a thin layer of PDMS between the microfluidic chip and the top sealing glass of the chip. That PDMS layer is elastic and will ensure the sealing of all the chambers.

13. To avoid the introduction of new bubbles inside the chambers, the microfluidic pump can be used to fill the microfluidic channels with complete media and empty those channels of air before the sealing step. If some small bubbles get stuck in the channels, applying pressure carefully on top of that channel can help to remove the bubbles. Alternatively, a peristaltic or a syringe pump can be used instead of the pressure pump.
14. It is important to ensure that the fluid is being pumped through the two inlets. In some cases, one of the inlets can be blocked, which causes a wrong gradient generation.

Competing Interests

JM is co-inventor of dynamic BH3 profiling (patented by Dana-Faber Cancer Institute) and has received royalties, was a paid consultant for Oncoheroes Biosciences and Vivid Biosciences, is an unpaid board member for the Society for Functional Precision Medicine, and is currently collaborating with AstraZeneca. JR-A and MAO are co-founders of Vitala Technologies and have stocks in the company; JR-A is Scientific Board member and MAO is a full-time employee in the company. No other relevant competing interests were disclosed by the other authors.

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Chapter 9

Kinetic Detection of Apoptosis Events Via Caspase 3/7 Activation in a Tumor-Immune Microenvironment on a Chip

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Abstract

The development of advanced biological models like microphysiological systems, able to rebuild the complexity of the physiological and/or pathological environments at a single-cell detail level in an in-vivo-like approach, is proving to be a promising tool to understand the mechanisms of interactions between different cell populations and main features of several diseases. In this frame, the tumor-immune microenvironment on a chip represents a powerful tool to profile key aspects of cancer progression, immune activation, and response to therapy in several immuno-oncology applications. In the present chapter, we provide a protocol to identify and characterize the time evolution of apoptosis by time-lapse fluorescence and confocal imaging in a 3D microfluidic coculture murine model including cancer and spleen cells.

Key words Micro-physiological systems, Organs-on-chip, Microfluidics, Cancer, Microenvironment, Apoptosis, Timelapse imaging, Fluorescence microscopy

1 Introduction

Our ability to dissect phenomena occurring in complex biological environments is strongly connected with the availability of biological models recapitulating the problem under study and to the possibility of measuring the processes that develop inside the system. "Advanced in vitro models" is the name that summarizes a family of approaches which focus on developing in-vivo-like models including cells and process occurring in animal models, moving them in an "under the microscope" configuration, enabling, thus, to multiply the access to relevant system describing parameters. In this context, the use of microfluidics to define the physical and chemical environment of the biological system –

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microphysiological systems (MPS) – is now one of the most promising technological advancements toward the reconstitution of physiologically relevant microenvironments in a high-throughput/high-content configuration [1, 2]. Moving cell cocultures, entire populations, or even functional units of organs onto microfluidic chips allows to perform multiple, parallel experiments, with the possibility to exploit all the potentiality of advanced microscopy along with standard biochemical techniques and artificial intelligence-enabled methods for processing high-content data [3–5]. These platforms can be employed to evaluate on one side cancer behavior and evolution [6, 7, 14] and, on the other hand, drug efficacy and mechanism of action fostering a personalized medicine approach by exploiting patients' biopsies [8–11].

The present work is aimed at highlighting the potentiality of on-chip methodologies combined with advanced microscopy to measure dynamically relevant biological processes, taking as scientific case apoptotic events occurring in a murine tumor microenvironment model upon immune cell coculture stimulus.

2 Materials

All solutions, reagents, and microfluidic devices used for cell culturing are sterile or sterilized. Prepare all reagents for collagen mix in ice and store at 4 °C. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Cell Culture and Collagen Preparation

1. MC-38 cell line (Kerafast).
2. Mouse splenocytes isolated from C57BL/6 mouse (Envigo RMS Srl) (*see* **Note 1**).
3. Complete DMEM: DMEM (Dulbecco's Modified Eagle Medium, Gibco, #11965–092) supplemented with 10% v/v fetal bovine serum (FBS, Gibco, Brazil, South America, #26140079), 1% penicillin/streptomycin (5000 U/mL, Gibco, #15070063), and 2 mM L-glutamine (Gibco #25030081).
4. Complete RPMI medium: RPMI (Roswell Park Memorial Institute, Gibco #11875093) 1640 supplemented with 10% v/v fetal bovine serum (FBS, Gibco, Brazil, South America, #26140079), 1% penicillin/streptomycin (5000 U/mL, Gibco, #15070063), and 2 mM L-glutamine (Gibco, #25030081).
5. TrypLE™ Express Enzyme 1X (Gibco, #12605010).
6. Trypan blue (Gibco #15250061).
7. Phosphate-buffered saline (PBS).
8. Hoechst 33342 (Invitrogen #H1399).

9. Collagen I (Gibco, #A1048301).
10. NaOH 1 N sterile solution.
11. Sterile 2dH2O.
12. CellEvent™ caspase-3/7 (Invitrogen #C10723).
13. Staurosporine (from *Streptomyces* sp., Sigma, #S6942-200ul) at a final concentration of 1 mM.
14. Anti-mCD3 (eBioscience, Invitrogen, #14–0031-82).
15. Cellvis 6-well glass-bottom plate (#P06–1.5H-N).

2.2 Equipment

1. Fully equipped cell biology facility with BSL2 biosafety cabinet and CO2 incubator.
2. Automated cell counter (alternatively, cell counting can be performed manually with a hemocytometer).
3. Reactive ion etching system.
4. Confocal microscope compatible with live cell imaging (stage-top incubator), equipped with 405- and 488-nm laser lines and transmitted light detector and relative filter cubes (DAPI and FITC) or spectral selection (multiwell plates are compatible with confocal imaging; *see* **Note 2**).

3 Methods

Microchips used for this protocol have been previously described as 3D immunocompetent tumor on chip model [8, 13]; *see* **Note 3.**

3.1 Microfluidic Chip Design and Process

1. Chips can be produced as PDMS replicas from photolithographically produced masters (Su-8 photoresist on silicon wafers). A detailed description of the fabrication steps can be found in previous work [8, 15].
2. Perform sealing of PDMS replicas on 6-well optical-bottom plates by oxygen plasma activation with the following process parameters: density power, 0.22 W/cm2; pressure, 0.8 Torr; and time, 35 s at 60 sscm oxygen flux. Parameters must be optimized depending on the machine used for functionalization (e.g., plasma cleaner) (*see* Fig. 1).

3.2 MC-38 Cell Culture

1. Culture cells in the complete DMEM.
2. Seed MC-38 cancer cell at 1×10^6 cells in a T75 flask and incubated at 37 °C in the humidified atmosphere with 5% CO2.
3. Aspire medium after the cells reached 85–95% confluence (doubling rate of this cell line is 12 h) and wash with PBS (8 mL for P75 flask).

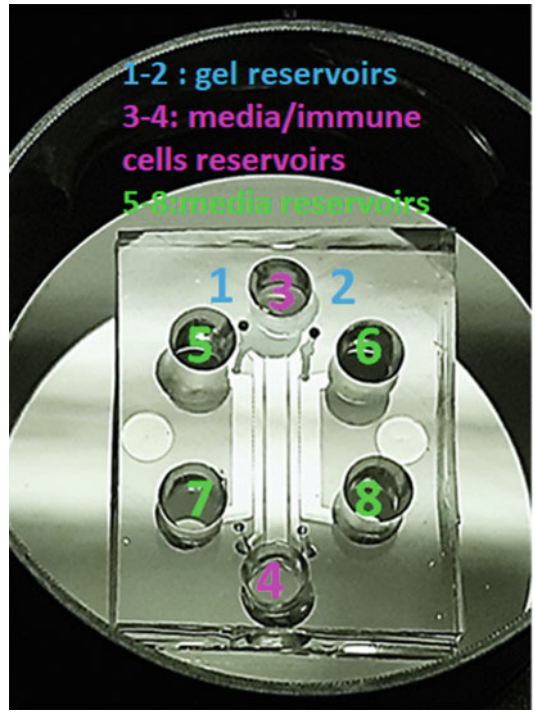

Fig. 1 Chip structure, with the indication of different compartments and medium reservoirs. The chip is bonded to an optical-bottom multiwell plate (*see* **Note 2**)

4. Detach cell monolayer using 2 mL TrypLE cell dissociation reagent for 2 min at 37 °C in the incubator.
5. Resuspend detached cells in complete growth medium.
6. Use via Trypan blue stain (20 μl cell suspension and 20 μl Trypan blue) to count cells and assess viability.
7. Take the volume of cell suspension corresponding to the number of cells needed: Final concentration of tumoral cells with this growth rate is 2000 cells/μl in hydrogel suspension. Total volume of each 3D chamber in this configuration is 3.5 μl, but it is suggested to prepare not less than 50 μl for each treatment sample to ensure homogeneous cellular suspension.

3.3 Nuclear Staining

1. Transfer cell suspension to a 1.5-mL vial.
2. Add 1:1000 sterile Hoechst 33342 (final concentration 10 μg/mL) to cell suspension and incubate in the dark at room temperature for 20 min.
3. Wash the cells with a complete medium (4 min, 1000 RPM, room temperature).

3.4 Collagen Preparation

1. In this protocol, type I collagen is used. The protocol is compatible with most hydrogel matrices for 3D cultures (optimization tests needed).
2. Prepare gel solution according to producer protocol (2 mg/mL final concentration): for 200 μl final gel solution 20 μl PBS 10X, 3,4 μl NaOH, 42,6 μl 2dH2O, 134 μl collagen. The pH has to be checked and adjusted to 7 (*see* **Note 4**).
3. Add the collagen solution to the cell pellet and suspend cells in the solution avoiding air bubble (perform a slow pipetting until complete mixing) formation.
4. Keep the cell suspension on ice (*see* **Note 5**).
5. Inject cells in collagen suspension into microchip inlets (1-mm diameter) slowly to fill the 3D chamber using a pre-cooled (kept at 4 °C) P10 tip (*see* **Note 6**).
6. Check with the microscope to verify absence of bubbles and complete filling of 3D channels (*see* **Notes 7** and **8**).
7. Place the multiwell in incubator at 37 °C for 20–30 min to allow for collagen polymerization.
8. Add culture medium to central and lateral reservoirs paying attention to adding the same medium amount in each reservoir (50–70 μl for each reservoir depending on reservoir dimensions).
9. Place the chip in an incubator (37 °C, 5% CO_2 in humidified atmosphere) for 24 h (*see* **Note 9**) (*see* Fig. 2).

3.5 Caspase 3/7 Assay

1. Add CellEventTM caspase-3/7 (Thermo Fisher Scientific cat no C10723) to the external reservoirs at a final concentration of 2 μM. Transfer cell suspension to a 1.5-mL vial.
2. Incubate the plate for 30 min in the dark at room temperature.

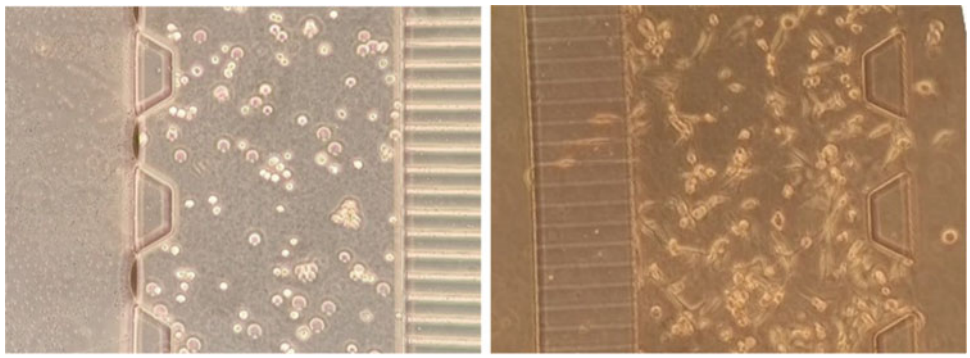

Fig. 2 Day 1: MC-38 cells and gel loaded before medium addition (**a**). Day 2: MC-38 cells after medium addition and 24-h culture (**b**) Magnification 10X

3.6 Splenocytes Cell Culture and Coculture

1. Culture cells in the complete RPMI medium and incubate at 37 °C in the humidified atmosphere with 5% CO2. For splenocyte preparation, *see* **Note 1**.
2. Incubate cells for 24 h with 1:100 anti mCD3 (eBioscience, Invitrogen, #14–0031-82) in 400 μl complete medium in a 24-multiwell plate.
3. After 24 h' incubation detach cells, and count via Trypan blue assay.
4. Consider 10:1 as splenocytes/MC-38 cell ratio for each chip and take the proper suspension volume to obtain the needed number of cells. In this experimental setting there were 140.000 splenocytes in each chip.
5. Spin down cells (1500 RPM, 5 min) and resuspend in a volume of 15 μl for each chip.
6. Aspire culture medium from reservoirs.
7. Add 7,5 μl of splenocyte suspension from each side of the central chamber and allow 10 min.
8. Check the position of immune cells at the microscope (*see* **Note 10**).
9. Add the same volume of medium to reservoirs.

3.7 Image Acquisition

1. Set incubation parameters (37 °C, CO_2 5% in humidified atmosphere) of microscope stage-top incubation system and allow the system to reach thermal equilibrium.
2. Place the multiwell plate in the adapter of the microscope stage.
3. Choose objective magnification and the fields of view according to the specificity of experiment. For this application, 10X objective is recommended (*see* Fig. 3).
4. Perform time-lapse acquisition using the following configuration: DAPI channel, nuclear counterstaining; FITC channel, apoptosis events; transmitted light, chip structure and cell morphological details. Set a total acquisition time of 12 h at 30-min intervals (*see* Fig. 4).

3.8 Image Analysis

1. Microscopy datasets can be analyzed by quantifying the number of apoptosis events, calculated as the ratio between the events recorded and quantified in the green channel divided by the total number of cells as indicated by the quantification of nuclei based on Hoechst staining, at predefined timepoints or following the kinetic evolution of the process. Transfer cell suspension to a 1.5 mL vial.
2. Add 1:1000 sterile Hoechst 33342 (final concentration 10 μg/mL) to cell suspension and incubate in the dark at room temperature for 20 min.
3. Wash the cells with a complete medium (4 min, 1000 RPM, Room Temperature).

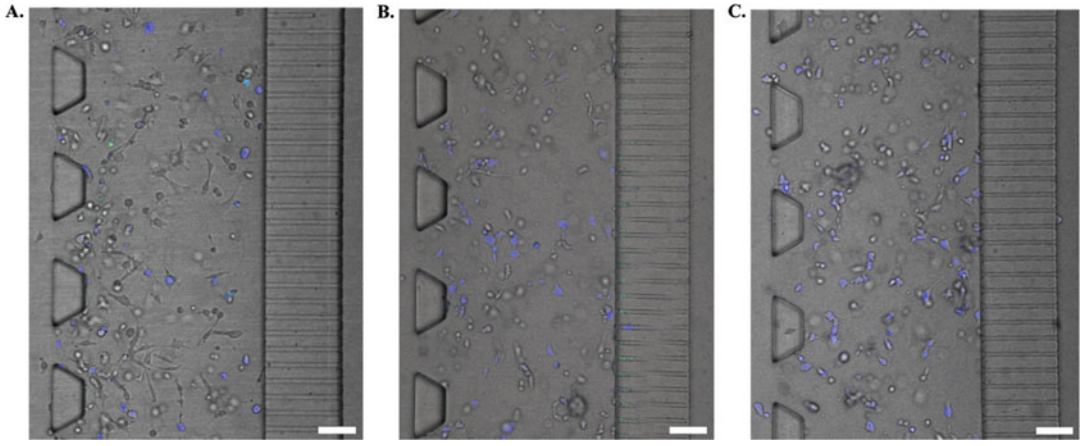

Fig. 3 Day 2: 2D images at the beginning of the time-lapse experiment (**a**) Negative control, (**b**) splenocyte coculture, and (**c**) STS (Staurosporine)-treated cells. Magnification 10X, dimensional bar 100 mm. The images are obtained merging bright-field channel, blue channel (Hoechst nuclear staining), and green channel (Caspase 3/7 activation marker)

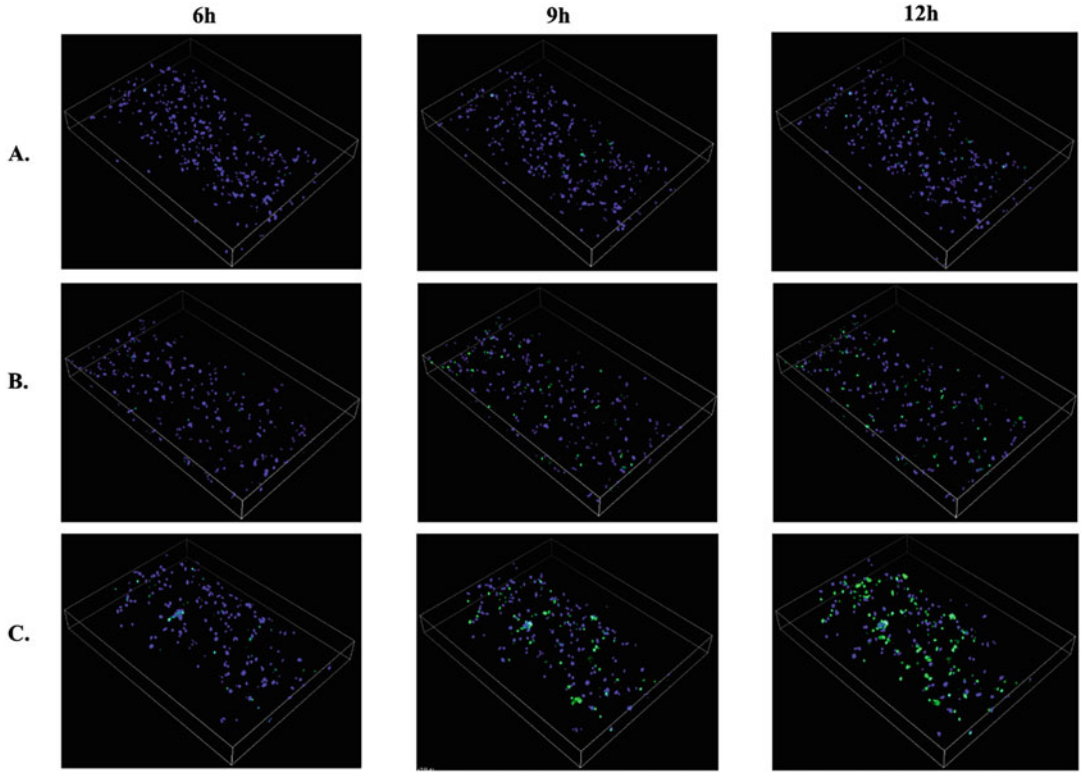

Fig. 4 Days 2–3: 3D images (Z stack projections) at 6, 9, and 12 h from treatment (**a**) Negative control, (**b**) Splenocyte coculture, (**c**) STS (Staurosporine)-treated cells. Magnification 10X. Blue, Hoechst nuclear stain, green, caspase 3/7 activation marker. Each stack is composed of 29 images, spaced 5 mm from each other, for a total thickness of 135 mm, XY imaged dimensions 1000 × 1273 mm

4 Notes

1. The splenocytes were isolated from C57BL/6 mice, previously subcutaneously injected in the right flank with 3×10^5 cells MC-38 colon adenocarcinoma cells in 0.1 mL of PBS and subsequently sacrificed according to protocol [12].
2. Glass = bottom thickness in this multiwell plate is compatible with confocal and two-photon excitation fluorescence microscopes and allow for the control of multiple experiments in series.
3. The chip consists of five major compartments: a central one for the floating immune cell intake, two side regions for embedding tumor cells in hydrogel matrices (150–250 μm high), and media perfusion chambers. Immune and tumor chambers are connected by two sets of narrow arrays of microchannels ($200 \times 12 \times 10$ μm^3, L × W × H). Regularly 100-μm-spaced trapezoidal isosceles micropillars (about 25–30 interfaces for each side gel region) work as barriers to confine gel solution during injection exploiting the balance between surface tension and capillary forces and connect tumor regions to the two lateral additional media chambers in order to set a gel–liquid interface.
4. Pre-cool all reagent collagen mixture and use pre-chilled pipette tips to avoid hydrogel pre-polymerization during mixing and injection procedure of solution in microchambers.
5. When mixing keep the pipette tip submerged in the solution and do not lift centrifuge tube from ice.
6. Use a fresh pipette tip to load each new gel channel to prevent bubbles trapping in the viscous solution within the channel.
7. Do not exert excessive pressure to load suspension to prevent collagen to leak out into adjacent channels.
8. When removing the micropipette from the gel inlets, keep firmly the plunger to avoid aspirating the gel solution.
9. Sterile water should be added in interstices located between wells to minimize medium evaporation during experiments.
10. After loading immune cells in the central chamber, if you note cells flowing directly inside gel chambers, discard the chip. This means that the gel is not correctly polymerized, disrupted, or detached.

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Chapter 10

Hypoxic 3D Tumor Model for Evaluating of CAR-T Cell Therapy In Vitro

Jeong Min Oh and Keyue Shen

Abstract

Solid tumors contain abnormal physical and biochemical barriers that hinder chimeric antigen receptor (CAR) T cell therapies. However, there is a lack of understanding on how the solid tumor microenvironment (e.g. hypoxia) modulates CAR-T cell function. Hypoxia is a common feature of many advanced solid tumors that contributes to reprogramming of cancer and T cell metabolism as well as their phenotypes and interactions. To gain insights into the activities of CAR-T cells in solid tumors and to assess the effectiveness of new combination treatments involving CAR-T cells, in vitro models that faithfully reflect CAR-T cell–solid tumor interactions under physiologically relevant tumor microenvironment is needed. Here we demonstrate how to establish a hypoxic 3-dimensional (3-D) tumor model using a cleanroom-free, micromilling-based microdevice and assess the efficacy of the combination treatment with CAR-T cells and PD-1/PD-L1 inhibition.

Key words Chimeric antigen receptor, CAR-T cell, Hypoxia, Immune checkpoint inhibition, Immunotherapy, Ovarian cancer, Solid tumors

1 Introduction

Adoptive chimeric antigen receptor (CAR) T cell therapy is a genetically engineered T cell therapy where T cells are transduced to express CAR, a synthetic receptor, to recognize tumor-associated antigens (TAAs) on the target cell surface. Such engagement activates the synthetic intracellular domains of the CAR involving T cell receptor (TCR) and costimulatory signaling, to elicit TAA-specific targeted cell killing [1]. CAR-T cells are highly effective against hematological malignancies [2], and to date (by April, 2022) there are six CAR-T cell therapies approved by the US Food and Drug Administration (FDA) to treat cancers including multiple myeloma, B cell lymphoma, and acute lymphoblastic leukemia. However, the efforts on translating CAR-T cell therapy against solid tumors have largely failed [3, 4]. Unlike hematological malignancies in a fluid landscape, solid tumors establish

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unique physical barriers in the tumor microenvironment (TME), such as a dense network of extracellular matrix (ECM) and elevated intratumoral fluid pressure, which hinder T cell infiltration [5]. In addition, CAR-T cells also have to overcome the biochemical barriers, i.e., the immunosuppressive cancer- and TME-derived factors like programmed death ligand-1 (PD-L1), which regulates the survival of T cells and compromise cytotoxicity of T cells against tumor cells [6]. Furthermore, hypoxia, a major hallmark of solid tumors, exacerbates immunosuppression in the TME as tumor cells upregulate the production of the inhibitory molecules (e.g., PD-L1) upon oxygen deprivation [7, 8]. Even so, there is still a lack of understanding on how the hypoxic TME modulates CAR-T cell function, to improve its therapeutic efficacy in solid tumors. The existing in vivo models are insufficient to address this problem, where the roles of hypoxia and different TME and systemic factors are difficult to delineate. They are also highly variable, labor intensive, and low-through in nature. Therefore, there is a significant need for in vitro hypoxic tumor models that allow for assessing CAR-T cell therapy and other combination treatments involving CAR-T cells under highly controlled but realistic physical and biochemical conditions.

We have lately established an in vitro tumor model of human ovarian cancer that enables high-content analysis of CAR-T-mediated cancer killing in a hypoxic solid TME [9]. We demonstrated that the model was suitable to investigate the immunosuppressive mechanisms of CAR-T actions in TME and to provide a fast-turnaround in vitro testing platform for combination treatments of CAR-T cell therapy and other regimens (e.g., PD-1/PD-L1 blockade). It was observed that PD-1/PD-L1 inhibition did not enhance the CAR-T-mediated cancer cell killing within the evaluated timescale, similar to the clinical studies using checkpoint inhibition [10].

Here we provide a detailed procedure on how to establish this in vitro tumor model in a research laboratory. In our model, cancer cells are embedded in an ECM hydrogel and cultured between two oxygen diffusion barriers in a micromilled microdevice. Oxygen consumption by cancer cells and limited replenishment lead to a naturally established oxygen and metabolic gradient within the cancer cell population in the device. A combinatorial treatment of HER2-targeting CAR-T cells and a PD-1/PD-L1 inhibitor is then introduced through the microfluidic channels surrounding the micropatterned tumor layer (Fig. 1). Overall, the platform mimics and evaluates CAR-T infiltration and cytotoxicity in hypoxic solid tumors with spatial and temporal resolution, which will facilitate the preclinical assessment of new combination CAR-T treatments before in vivo experiments.

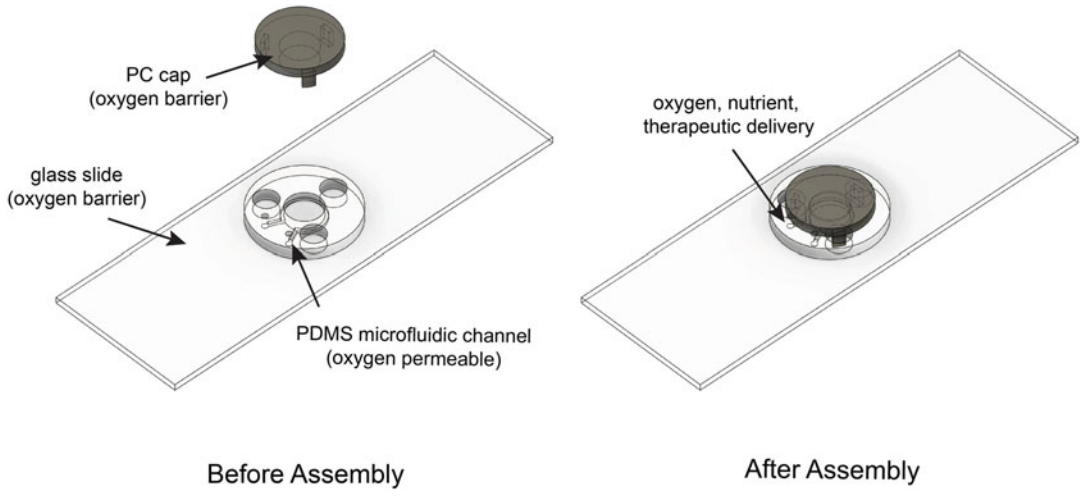

Fig. 1 Schematics of the hypoxic microdevice before and after assembly. A PDMS chamber/channel is plasma-bonded to a clean glass slide (the first oxygen barrier). A micromilled polycarbonate (PC) cap is tightly assembled on top of the PDMS chamber/channel to serve as the second oxygen barrier on top of a 3-D hydrogel embedded layer of cancer cells. An oxygen gradient is generated by cancer cell metabolism and limited diffusion/replenishment

2 Materials

Sterilize all dry lab materials with 70% ethanol bath or by autoclaving before using them for cell culture. Practice aseptic laboratory technique when preparing solutions and reagents used for cell culture. Perform all cell culture work in a sterile biosafety cabinet with proper personal protective equipment (PPE). Incubate cells in a humidified 37 °C, 5% CO_2 incubator.

2.1 Hypoxia Microdevice Design and Fabrication

1. Computer-aided design (CAD) software: Autodesk Fusion360 (Autodesk Inc.).
2. Desktop computer numerical control (CNC) milling machine and machine control software: Nomad 883 Pro (Carbide 3D) and Carbide Motion (Carbide 3D), 1/16″ and 1/32″, 2-flute flat end mill (Bantam Tools), double-sided tape (Scotch), and vacuum for proper debris cleaning and machine maintenance.
3. Polycarbonate (PC) stock material: 4″ × 5″ × 0.236″ polycarbonate (Bantam Tools).
4. Vapor polishing: hot plate, dichloromethane (Sigma-Aldrich), aluminum foil, and tweezers. The protocol for vapor polishing is obtained from Yen et al. [11].
5. Polydimethylsiloxane (PDMS): 10:1 base to curing agent mixture of Dow Corning SYLGARD 184 Silicone Elastomer.

6. Biopsy punch: Φ1.0 mm, Φ4.0 mm, and Φ6.0 mm Limited Reuse Biopsy Punch (World Precision Instruments).
7. Clean glass slide for cell culture: Place plain 1″ × 3″ microscope slides (Globe Scientific) on a glass slide rack (Electron Microscopy Sciences). Pour 250 mL of ES-7X cleaning solution (MP Bio) and 750 mL of Milli-Q water (Millipore Sigma) into a 10,000-mL glass beaker. Submerge glass slide rack in the solution and place a magnetic stirring bar into the beaker. Wrap aluminum foil on top of the beaker before placing the beaker on a hot plate with magnetic stirrer and temperature probe. Set the target temperature at 80 °C with a heat probe in the solution and stir at 200 RPM until the solution turns clear (typically 30 min at target temperature). Remove glass slide rack and rinse thoroughly by completely refilling 10 times with deionized water and finish with 1× Milli-Q water. Remove water, dry glass slides with air, and bake in a furnace at 400 °C for 6 h to remove all remaining organic material. Allow the glass to cool to room temperature before use.
8. High-power expanded plasma cleaner for plasma bonding: model PDC-001-HP (Harrick Plasma).

2.2 Establishing 3D Hypoxic Tumor Model

1. Cancer cell: SKOV3 human epithelial ovarian cancer cells (ATCC) cultured in a T75 flask, in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher) supplemented with 10% v/v fetal bovine serum (Sigma-Aldrich), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (P/S, Thermo Fisher), 4×10^{-3} M sodium pyruvate (Fisher Scientific), and 2×10^{-3} M GlutaMAX (Thermo Fisher). Store DMEM at 4 °C.
2. Hydrogel constituents: 250 μL of 20% w/v gelatin methacryloyl (GelMA; 8% MA) solution in sterile phosphate-buffered saline (PBS, 1×; VWR), store at 4 °C. 75 μL of 2% w/v lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) in sterile PBS, store at −80 °C. Prepare 10 mL of 10× concentrated DMEM by mixing 1.348 g of DMEM powder (Thermo Fisher), 0.494 mL of 7.5% w/v sodium bicarbonate (Thermo Fisher), and 9.51 mL of Milli-Q water (Millipore Sigma). Adjust the pH of the 10× DMEM to 7 and filter through 0.2-μm Nalgene sterile syringe filters (Fisher Scientific), before storing it at 4 °C. Sterile 1× Ca^{2+}-free and Mg^{2+}-free PBS (VWR); store at room temperature.
3. Sterile 1× Ca^{2+}-free and Mg^{2+}-free PBS (VWR) for cell harvesting; store at room temperature.
4. 1× trypsin/EDTA solution (0.05%): Dilute 5 mL of 10× trypsin–EDTA (0.5%; Thermo Fisher) with 45 mL of 1× Ca^{2+}-free and Mg^{2+}-free PBS (VWR). Store at 4 °C.

5. Vortex mixer.
6. Sterile 100 × 15 mm and 150 × 15 mm (diameter x height) petri dish.
7. Sterile 0.65-mL microcentrifuge tubes.
8. Sterile 15-mL and 50-mL centrifuge tubes.
9. Filtered ultraviolet (UV) light source that can emit UV wavelength of 375 ± 14 nm with intensity of at least 2.34 ± 0.26 mW cm^{-2} for 120 s.
10. Wet chamber for microdevice (this will house the microdevice in the incubator): This is prepared in a sterile biosafety cabinet. Spray Kimwipes (Kimberly-Clark Professional) with 70% IPA and place them in the biosafety cabinet to dry. Fold them and place them against the inner wall of a sterile 150 × 15 mm petri dish. Soak the folded Kimwipes with sterile water. The inner walls of the sterile petri dish should be fully surrounded by soaked Kimwipes. Be careful not to flood the petri dish and aspirate excess sterile water. Close lid and store inside the biosafety cabinet until use.

2.3 Combination CAR-T and PD-1/PD-L1 Inhibition Therapy

1. T cells and CAR-T cell culture: Human T cells are isolated from peripheral blood mononuclear cells (PBMCs). Lentiviral particles carrying the anti-HER2 (4D5) scFv CAR construct are stored at −80 °C. Transduce T cells with CAR lentivirus following Siegler et al. [12]. Prepare T cell medium by supplementing X-VIVO 15 serum-free medium (Lonza) with 5% v/v GemCell human serum antibody AB (Gemini Bio-Products), 2×10^{-3} M GlutaMAX (Thermo Fisher), 10×10^{-9} M HEPES buffer (Corning), 1% v/v P/S (Thermo Fisher), 12.25×10^{-3} M *N*-acetyl-L-cysteine (Sigma-Aldrich), and 10 ng mL^{-1} human IL-2 (Peprotech). Store T cell medium at 4 °C.
2. PD-L1 blockade: Prepare a stock solution (53.99 mM) by adding 1 mL of sterile water to the 100 mg PD-1/PD-L1 Inhibitor 3 powder (Selleck Chemicals, IC50 = 5.6×10^{-9} M). Store stock solution at −80 °C. Prepare working solution by diluting the stock solution to 10×10^{-9} M in fresh T cell media.

2.4 Cytotoxicity Assay

1. Live/dead staining solution: calcein-AM (Thermo Fisher) for live staining and propidium iodide (PI; Thermo Fisher) for dead staining. Prepare staining solution by diluting calcein-AM and propidium iodide to a working concentration (5 μM for each stain) in cell culture media.
2. Sterile 1× Ca^{2+}-free and Mg^{2+}-free PBS (VWR), store at room temperature.

3. Single-edge razor blade for complete microdevice disassembly.
4. Sterile 100 × 15 mm (diameter x height) petri dish.

2.5 Immunostaining

1. 4% paraformaldehyde (PFA): Dilute 10% PFA solution (Electron Microscopy Sciences) to make 4% v/v PFA in 1× PBS.
2. 0.1% v/v Triton X-100 (Fisher Scientific) in 1× PBS.
3. 4% bovine serum albumin (BSA): Dissolve BSA (GE Healthcare Biosciences) in 1× PBS (4% w/v) and filter through 0.2-μm Nalgene sterile syringe filters (Fisher Scientific), before storing it at 4 °C.
4. FluoroGel II containing 4′,6-diamidino-2-phenylindole (DAPI) (Electron Microscopy Sciences).
5. Primary antibody: anti-CD45 (HI30; 1:200; Thermo Fisher).
6. Secondary antibody: Donkey anti-Mouse Alexa Fluor 647 (1:500; Thermo Fisher).
7. Glass coverslips.

2.6 Microscopy

1. A Nikon Eclipse Ti-E inverted fluorescence microscope (Nikon).
2. Nikon C2 point-scanning confocal microscope (Nikon).

3 Methods

3.1 Hypoxia Microdevice Design and Fabrication

1. Create CNC milling toolpaths for the hypoxia microdevice (a cap structure and the master mold for the PDMS chamber/channel) using Autodesk Fusion360 (*see* **Notes 1** and **2**).
2. Export toolpath by converting toolpath into a machine code (GRBL for milling on Nomad 883 Pro).
3. Turn on the CNC milling machine and connect to a machine control software (Carbide Motion) for initialization. Attach a 1/16″, 2-flute flat end mill to the spindle of the machine.
4. Apply double-sided scotch tape to the bottom surface of the polycarbonate stock material and place polycarbonate stock material on top of the milling platform. Press down across the surface of the polycarbonate stock material so that it can adhere to the milling platform (*see* **Note 3**).
5. Calibrate the machine and ensure that the coordinates recognized by the machine match the coordinate of the toolpath (*see* **Note 4**).
6. Import the converted toolpath code into the machine control software (Carbide Motion).
7. Begin the initial milling process.

8. After completing the first milling process, clean up the milling platform by vacuuming the polycarbonate debris (*see* **Note 5**). Replace the 1/16″, 2-flute flat end mill with 1/32″, 2-flute flat end mill for the second toolpath. Re-initialize and recalibrate the machine, if needed.
9. Begin the final milling process.
10. After the completing the final milling process, clean up the polycarbonate debris using a vacuum. Gently pry off the milled polycarbonate from the platform. Repeat the milling process for each microdevice component (the cap and the master mold). Remove all debris from the milled components before vapor polishing (*see* **Note 6**).
11. All vapor polishing procedure is conducted inside a chemical fume hood. Pour 10 mL of dichloromethane into a Buchner flask and cover the top of the flask with aluminum foil (*see* **Note 7**). Place flask on top of a hot plate and set hot plate to 80 °C. Wait until a steady vapor stream is observed from the hose nozzle.
12. Once a steady vapor stream is observed, pick up a milled component with a tweezer and pass several times through the vapor stream until all surface of the milled component is optically transparent (*see* **Note 8**). Repeat for both components.
13. Leave vapor polished components in the fume hood overnight to ensure that all vapor is evaporated. Rinse with water and dry before handling. Sterilize the polycarbonate cap before using them for cell culture.
14. Use compressed filtered air to gently remove particles from the master mold. Pour uncured PDMS mixture (10:1 base to curing agent) into the master mold (*see* **Note 9**) and cure overnight at 65 °C.
15. Remove cured PDMS from the master mold. Use the 1.0-mm biopsy punch to open the inlet and outlet of the microfluidic channel. Use the 6-mm biopsy punch to remove the thin layer of PDMS at the center of the PDMS chamber/channel. Use the 4-mm biopsy punch to create three evenly spaced holes surrounding the 6-mm hole at the center (*see* **Note 10**). Clear debris on the surface of PDMS using tape.

3.2 Establishing 3D Hypoxic Tumor Model

1. The hypoxia gradient under cell culture conditions has been characterized and validated through silica microparticle oxygen sensors and immunostaining for Glut-1, an intrinsic cellular marker of hypoxia [9]. The mechanical property of the tumor model has been characterized using a uniaxial mechanical compression test [9]. Sterilize all materials that will be in direct contact with the cells during these procedures. All procedures

involving cells are performed in a sterile biosafety cabinet. Pre-warm cell culture reagents and media at 37 °C.

2. Plasma-bond prepared PDMS device onto a clean glass slide (*see* **Note 11**).
3. Sterilize bonded PDMS–glass slides with UV radiation for at least 30 min.
4. Immediately place PDMS–glass slides in a sterile 150 × 15 mm cell culture dish and transport into a biosafety cabinet (*see* **Note 12**).
5. Mix 75 μL of LAP with 250 μL of GelMA using the vortex mixer to create a working GelMA solution. Keep solution at 37 °C after mixing.
6. Transport T75 flask containing SKOV3 cells into the biosafety cabinet. Remove the culture medium and wash the cells with 1× Ca^{2+}-free and Mg^{2+}-free PBS.
7. Add 2 mL of pre-warmed trypsin/EDTA to the flask and incubate in a humidified 37 °C, 5% CO_2 incubator for 4 min, or until cells detach from the surface of the flask.
8. Add 6 mL of cell culture media to inactivate trypsin activity and transfer cell solution into a 15-mL centrifuge tube. Centrifuge solution at 200 × *g* for 5 min.
9. Carefully remove supernatant without disrupting the cell pellet and resuspend the cell pellet with 1 mL of pre-warmed cell culture media.
10. Count cells and pellet 1×10^7 cells in a 0.65-mL microcentrifuge tube by centrifugation at 200× *g* for 5 min.
11. Remove supernatant and resuspend the cell pellet with the following: 24.6 μL of 1× Ca^{2+}-free and Mg^{2+}-free PBS, 10 μL of 10× DMEM, 65 μL of working GelMA solution (*see* **Note 13**). Adjust the final pH of the cell-laden solution to ≈7.
12. Hold a sterilized polycarbonate cap so that the surface of the central pillar faces up, and pipette 4 μL of cell-laden GelMA solution onto the surface of the central pillar (*see* **Note 14**).
13. Flip the polycarbonate cap so that the surface of the central pillar (with the droplet of GelMA solution) faces down, and immediately assemble the polycarbonate cap into the PDMS–glass slide (*see* **Note 15**).
14. Place the assembled microdevice in a sterile 100 mm × 15 mm petri dish, and transport to a filtered UV light source (*see* **Note 16**).
15. Cross-link GelMA by exposing the microdevice to UV light (wavelength of 375 ± 14 nm) for 120 s at 2.34 ± 0.26 mW cm^{-2}.

16. Transport microdevice back into a biosafety cabinet (*see* **Note 17**).
17. Flush the PDMS channel of the microdevice with 1× PBS to remove excess uncured GelMA and fill with pre-warmed cell culture media (~20 μL of media per microdevice).
18. Place microdevice in the wet chamber and place wet chamber in a humidified 37 °C, 5% CO_2 incubator (*see* **Note 18**).

3.3 Combinatorial CAR-T and PD-1/PD-L1 Inhibition Therapy

1. Microdevices are incubated under normoxic (PDMS plug; *see* **Note 15**) or hypoxic (polycarbonate cap) conditions for 24 h before loading treatment(s) to the microfluidic channel of the microdevice. All procedures involving cells are performed in a sterile biosafety cabinet. Pre-warm cell culture reagents and media at 37 °C.
2. Transport T75 flask containing CAR-T cells into a biosafety cabinet. Transfer the cell solution from the flask into a 50-mL centrifuge tube. Centrifuge solution at 300× *g* for 5 min.
3. Carefully remove the supernatant, resuspend the pellet, and count cells. Pellet 8×10^5 CAR-T cells for each incubated microdevice by centrifugation at 300× *g* for 5 min (*see* **Note 19**).
4. Remove supernatant and resuspend the cell pellet with the working solution of PD-L1/PD-1 inhibitor 3 to achieve a CAR-T cell density of 40 million cells mL^{-1} (*see* **Note 20**).
5. Transport wet chamber containing the microdevices into the biosafety cabinet.
6. Remove culture media from the microdevice and load combination treatment solution. Ensure that the polycarbonate cap/PDMS plug is properly assembled to the PDMS–glass slide.
7. Place microdevice in the wet chamber and place wet chamber in a humidified 37 °C, 5% CO_2 incubator.

3.4 Evaluating the Therapeutic Efficacy of PD-L1 Blockade and CAR-T Cell Combination Treatment

3.4.1 Cytotoxicity Assay

1. Micropatterned cancer cells are treated with the combination therapy for 24 h. The therapeutic efficacy of the combinatory therapy is evaluated based on the cytotoxicity of the treatment and CAR-T cell infiltration into the 3D micropatterned tumor [9]. All procedures involving cells are performed in a sterile biosafety cabinet.
2. Pre-warm live/dead staining solution and cell culture reagents at 37 °C.
3. Transport wet chamber containing the microdevices into the biosafety cabinet.

4. Remove combination treatment solution and flush the microfluidic channel with fresh media.
5. Disassemble the microdevice by removing the polycarbonate cap/PDMS plug from the PDMS–glass slide. Remove the PDMS chamber/channel from the glass slide using a single-edge razor blade (*see* **Note 21**).
6. Aspirate remaining media around the micropatterned tumor and add 100 μL of the live/dead staining solution on top of the micropatterned tumor (*see* **Note 22**). Place micropatterned tumor back in the wet chamber and incubate in the biosafety cabinet at room temperature for 30 min.
7. Aspirate live/dead staining solution and place glass slide in a 100 mm × 15 mm petri dish.
8. Fill up the 100 mm × 15 mm petri dish with 1X PBS so that the entire glass slide is fully submerged (*see* **Note 23**).
9. Image for analysis (*see* **Note 24**).

3.4.2 CAR-T Cell Infiltration

1. After imaging for cytotoxicity, the CAR-T cell infiltration into the micropatterned tumor bulk can be assessed through immunostaining. Immunostaining is performed in a chemical fume hood. Ensure that the micropatterned tumor is entirely covered by the added staining solution.
2. Remove glass slide from the PBS-filled petri dish and wipe away excess PBS from the glass slide.
3. Remove remaining PBS around the micropatterned tumor and add 100 μL of the 4% PFA on top of the micropatterned tumor.
4. Incubate for 30 min at room temperature.
5. Remove 4% PFA and wash with PBS.
6. Remove PBS and add 100 μL of the 0.1% Triton X-100 on top of the micropatterned tumor.
7. Incubate for 30 min at room temperature.
8. Remove 0.1% Triton X-100 and wash with PBS.
9. Remove PBS and add 100 μL of the 4% BSA solution on top of the micropatterned tumor.
10. Incubate for 2 h at room temperature.
11. Remove 4% BSA solution and add 60 μL of the diluted anti-CD45 primary antibody solution (1:200) on top of the micropatterned tumor.
12. Incubate overnight at 4 °C.
13. Remove primary antibody solution and wash with PBS three times for 5 min per PBS wash. After the final wash, add 100 μL of 4% BSA solution to the micropatterned tumor.

14. Remove 4% BSA solution and add 60 μL of the diluted donkey anti-Mouse Alexa Fluor 647 secondary antibody solution (1: 500) on top of the micropatterned tumor.
15. Protect from light and incubate for 2 h at room temperature.
16. Remove secondary antibody solution and wash with PBS three times for 5 min per PBS wash.
17. Remove PBS and cover the micropatterned tumor with FluoroGel II containing DAPI.
18. Protect from light and incubate for 30 min at room temperature.
19. Place a glass coverslip on top of each micropatterned tumor.
20. Image for analysis (*see* **Note 25**).

4 Notes

1. The design for the hypoxia microdevice is created in Autodesk Fusion360 (Fig. 2) and fabricated using Nomad 883 Pro milling machine and software. However, other CAD software and other CNC milling machine and software may also be utilized, as long as the design resolution of the hypoxia microdevice can be achieved. The microdevice consists of (1) a cap structure with a central pillar (6-mm diameter) and three reference pillars that determine the gap size for oxygen diffusion (e.g., the central pillar is 100 μm shorter than the reference pillars) and (2) a PDMS chamber/channel, which is plasma-bonded to (3) a clean glass slide. The cap is tightly assembled with the PDMS chamber during hypoxic tumor (Fig. 3). The spatial and temporal profiles of oxygen expected in this device design has been shown through COMSOL Multiphysics simulation [9].
2. For each component, the "adaptive clearing" roughing strategy is utilized to create the milling toolpaths. Separate toolpaths are created for each flat end mill that is used in the fabrication process (e.g., 1/16″ and 1/32″ flat end mill had its own toolpath). Larger flat end mill is utilized for quick polycarbonate stock removal and the smaller flat end mill is utilized for detail.
3. Double-sided scotch tape is not needed, if the milling platform has alternative methods of securing the stock material. The stock material can break during milling, if the material is not adhered well to the platform.
4. This is process is referred to as "zeroing" the machine. This informs the machine where the stock material is located and where the milling should begin.

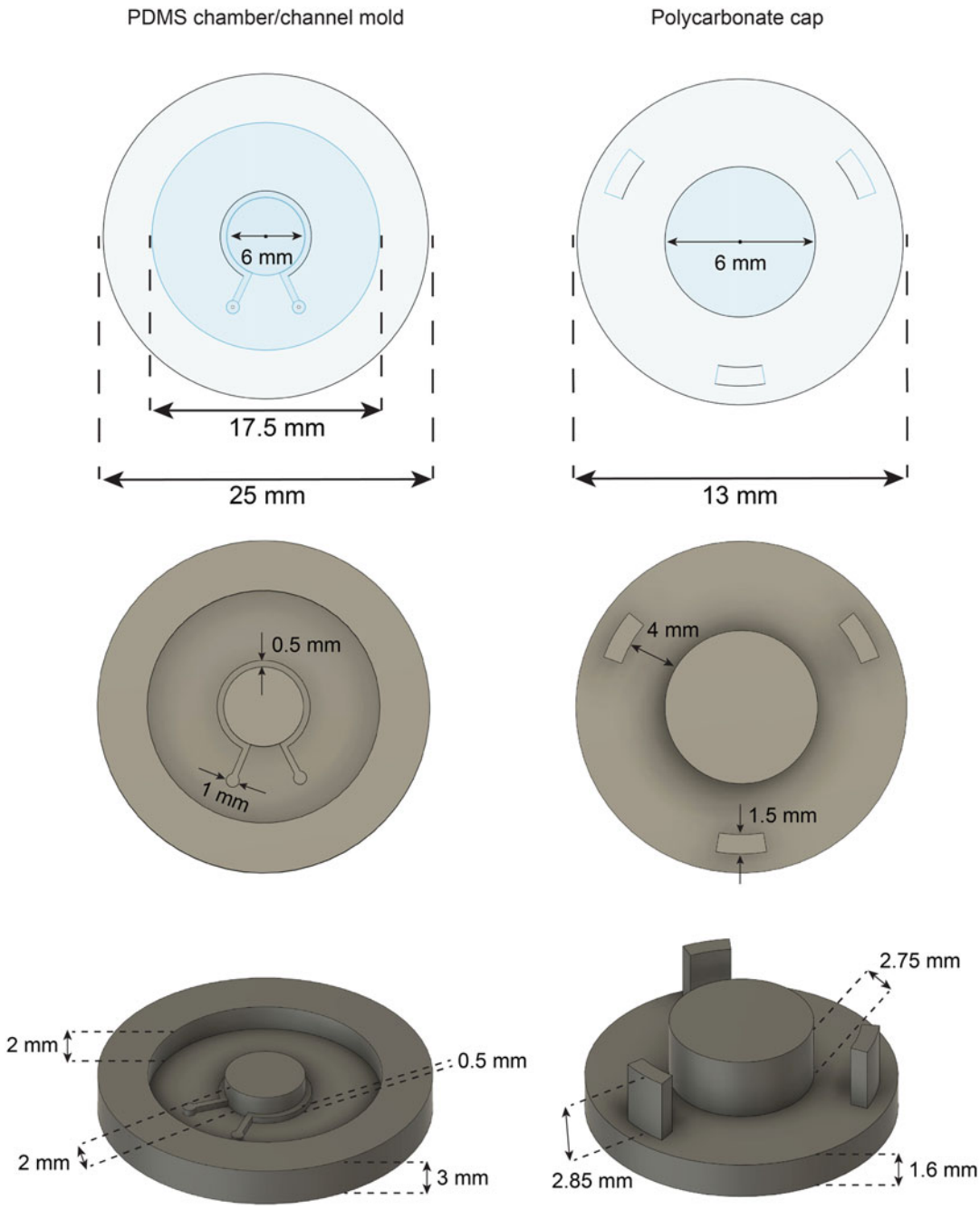

Fig. 2 CAD design and dimension of the components that are needed to fabricate the hypoxia microdevice. To fabricate the hypoxia microdevice, a master mold for the PDMS chamber/channel and the cap must be milled. Both components are produced with polycarbonate

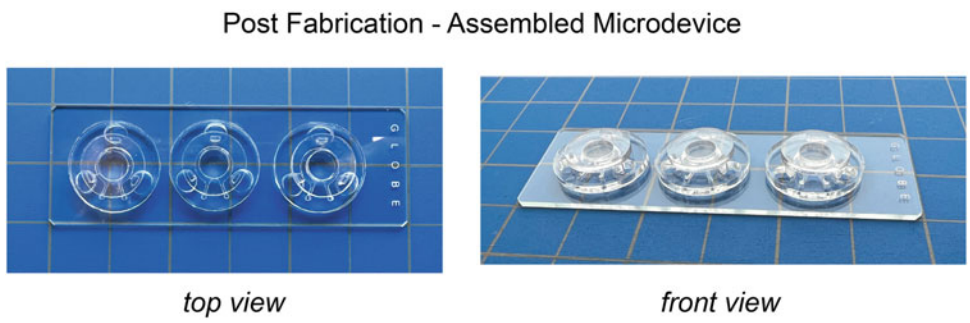

Fig. 3 Images of the assembled hypoxia microdevice post-fabrication

5. Avoid directly vacuuming the milled component as it may shift away from its original position. It is crucial that the location of the milled component is undisturbed for the accurate milling of the second toolpath.
6. Chemical vapor polishing without removing polycarbonate debris will embed unwanted debris onto the milled component.
7. Covering the top of the flask forces dichloromethane vapor to escape through the narrow hose nozzle, which produces a steady vapor stream.
8. Ensure that proper PPE is worn during vapor polishing as dichloromethane gas is toxic. Avoid making direct contact with the hose nozzle during polishing. We find that it is best to maintain a distance of ~1 cm between the milled component and the hose nozzle. Extended exposure (>1 s of continued exposure) to the vapor stream may disrupt the intended design of the microdevice. Be cautious when handling the milled component directly after vapor polishing as the polished surface will be softened and highly prone to scratches.
9. Remove all bubbles from the PDMS mixture using a vacuum desiccator before curing.
10. Carefully examine figures showing the punched PDMS chamber before punching (Figs. 1 and 3). Then 6-mm and 4-mm holes are positioned so that the polycarbonate cap can fit at the center of the PDMS chamber/channel during microdevice assembly. Verify that all three reference pillars of the polycarbonate cap can be placed through the 4-mm holes without stretching the PDMS chamber/channel. Improper positioning of the 4-mm holes will hinder proper microdevice assembly and will disrupt hypoxia induction.
11. We allow the plasma cleaner to be powered on at least 15 min prior to each plasma treatment. For model PDC-001-HP plasma cleaner (Harrick Plasma), PDMS and glass slides are treated at “low” power for 1 min. Contact plasma-treated

surfaces immediately after plasma treatment. Ensure that the entire surface of the PDMS is fully bonded to the glass slide. Incomplete bonding may disrupt the structure integrity of the microfluidic channel and cause leakage. Perform plasma bonding at a clean work area to prevent particles from entering the microdevice.

12. We find that a maximum of two glass slides per petri dish is best for handling.
13. The viscosity of the working GelMA solution serves as a challenge in achieving a homogenous cell-laden GelMA solution. We find that it is best to initially add PBS and 10X DMEM to the cell pellet and manually resuspend the cell pellet using a pipette before adding the working GelMA solution. We find that it is best to use a vortex mixer to ensure that the cells are distributed homogenously within the solution. Take extreme caution when using the vortex mixer for resuspension as it may damage the living cells. We find that it is best to set the vortex mixer in "touch mode" and mix for a short duration (~1 s) as frequently as needed.
14. GelMA solution should be at the center of the central pillar. Four microliters of the prepared GelMA solution is ~40,000 cells. Up to 24 microdevices can be established with the prepared GelMA solution.
15. **This step is the most crucial step in establishing the hypoxic tumor model.** As it was described in **Note 9**, the three reference pillars should be placed in the 4-mm holes and the central pillar should be placed in the 6-mm hole at the center of the PDMS. Push down on the polycarbonate cap so that all three reference pillars are touching the glass slide and ensure that the cap is securely assembled to the PDMS. Also confirm that the GelMA solution is spread evenly across the central pillar and is fully occupying the gap between the central pillar and the glass slide. Aspirate and re-pipette the GelMA solution until a bubble-free, evenly distributed, GelMA micropattern is achieved. If the cap is not fully secured to the PDMS, the cap may disassemble after adding culture media, which will disable the induction of hypoxic gradient in the tumor model.
16. The specified size of the sterile petri dish is appropriate for our UV light platform. Other sterile methods of transportation can be utilized.
17. Upon crosslinking, the polycarbonate cap is either kept assembled for hypoxic culture or disassembled and replaced with a 6-mm biopsy-punched PDMS plug for normoxic culture.
18. The wet chamber fully ensures that the microdevice does not dry out.

19. CAR-T cells are loaded at a 20:1 effector/target (E:T) ratio to each microdevice (~40,000 target cells are assumed to be in each 3D micropatterned tumor). Therefore, 8×10^5 CAR-T cells should be loaded in each microdevice.
20. This mixture of PD-1/PD-L1 inhibitor and CAR-T cell is the combination treatment solution. Each microdevice can hold ~20 μL of the combination treatment solution.
21. **Be extremely cautious during the PDMS removal as the razor blade can damage the micropatterned tumor.** We find it best to position the blade at a ~ 20° angle from the glass slide, when making the cut. When >50% of the PDMS–glass contact surface has been cut, peel the PDMS chamber/channel using hands. However, a more thorough cut may be needed depending on the strength of the plasma bond. Only the micropatterned tumor (crosslinked cell-laden GelMA) should remain on the glass slide after the complete disassembly.
22. The live/dead staining solution should fully cover the micropatterned tumor to ensure that (1) it does not dry out and (2) the entire micropattern is stained.
23. The micropatterned tumor is submerged in 1× PBS for imaging (i.e., we are providing a homogenous refractive index). It is not necessary to fill up the petri dish completely with PBS. We find it best to fill up half of the petri dish with PBS to prevent any handling mistakes (e.g., overflowing and spillage).
24. Images can be analyzed using the ImageJ, Python, and MATLAB software. Cytotoxicity is quantified by counting the number of identified PI-positive cells (dead cells) in the micropatterned tumor. Location of the dead cells are referenced to the centroid of each micropattern, are binned into 100 radially evolving concentric circles, and are plotted against micropattern radii. Additional information regarding the results can be found in our study [9].
25. Images can be analyzed using the ImageJ, Python, and MATLAB software. Infiltration is quantified by measuring the distance from the centroid of the CD45+ cells to the centroid of the nearest GelMA-embedded SKOV3 cancer cell or the nearest GelMA boundary. Additional information regarding the results can be found in our study [9].

Acknowledgement

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Chapter 11

Rapid Screening of CAR T Cell Functional Improvement Strategies by Highly Multiplexed Single-Cell Secretomics

Dragana Slavkovic-Lukic, Jessica Fioravanti, Azucena Martín-Santos, Edward Han, Jing Zhou, and Luca Gattinoni

Abstract

The functional fitness of CAR T cells plays a crucial role in determining their clinical efficacy. Several strategies are being explored to increase cellular fitness, but screening these approaches in vivo is expensive and time-consuming, limiting the number of strategies that can be tested at one time. The presence of polyfunctional CAR T cells has emerged as a critical parameter correlating with clinical responses. However, even sophisticated multiplexed secretomic assays often fail to detect differences in cytokine release due to the functional heterogeneity of CAR T cell products. Here, we describe a highly multiplexed single-cell secretomic assay based on the IsoLight platform to rapidly evaluate the impact of new pharmacologic or gene-engineering approaches aiming at improving CAR T cell function. As a key study, we focus on CD19-specific CAR $CD8^+$ T cells modulated by miR-155 overexpression, but the protocol can be applied to characterize other functional immune cell modulation strategies.

Key words Immunotherapy, Polyfunctionality, CAR T cells, T cell fitness, miR-155

1 Introduction

In the last decade, immunotherapy has become the fourth pillar of cancer treatment. Adoptive T cell therapy (ACT), using either naturally occurring tumor-infiltrating lymphocytes (TILs) or T cells genetically engineered to express T cell receptors (TCR) or chimeric antigen receptors (CAR) designed to recognize tumor antigens, has greatly improved the prognosis of many cancer patients resistant to standard chemo- and/or radiotherapy [1–4]. However, numerous patients do not benefit from these treatment modalities [1, 2]. Several factors have been implicated in T cell therapy failure, including antigen escape [5], inefficient trafficking of T cells to tumor tissues [6], and the presence of multiple immunosuppressive signals within the tumor-microenvironment [7, 8]. Additionally, the differentiation state and fitness of

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transferred T cells have been demonstrated to be key parameters determining the overall efficacy of ACT [9, 10].

Particularly, early memory T cell subsets endowed with stem cell-like attributes have shown remarkable activities in preclinical mouse tumor models [11–14] and correlate with objective responses in CAR-T [15–17], TCR-T [18], and TIL therapies [19]. These stem cell-like T cells are characterized by robust proliferative capacity, extreme longevity, and polyfunctionality (i.e., the capacity of T cells to simultaneously produce multiple cytokines) [11, 14, 20–22]. Ongoing efforts are being devoted to develop new methods to increase T cell stemness and functional fitness with the goal of potentiating the ACT efficacy. Strategies include the modulation of T cell metabolism [23–26], signaling pathways [11, 27–29], miRNAs [30–32], transcription factors [33–35], and epigenetic regulators [36–38]. Numerous candidates are in the pipeline of both research institutes and biotech companies. Evaluating these molecules in vivo in mouse models represents the ultimate test to determine their translational potential but these experiments are costly and time-consuming. In vitro surrogates for in vivo efficacy are therefore warranted, as there is only a limited number of strategies that can be tested at one time. Polyfunctionality has recently been shown to significantly segregate responders from patients experiencing progressive disease after CD19-specific CAR T cell therapy [39]. The heterogeneity of T cell products, however, can often mask differences in cytokine release when determined at bulk levels. In this chapter, we will provide a detailed protocol for a highly multiplexed single-cell secretomic assay based on the IsoLight automated platform. The assay analyzes up to 32 different cytokines produced by a single cell. Each sample is loaded on a single IsoCode chip where single cells are incubated in individual microchambers, and secreted cytokines are captured by preloaded antibodies (*see* Fig. 1). Spatial location of the proteomic barcode is used to detect signal intensity resulting from each cytokine. As an example, we will use miR-155 overexpression technology, which we have previously shown to boost T cell therapy in vivo [30, 31, 36]. We will show how this assay can be used to reveal differences in T cell polyfunctionality with unprecedented resolution that could not be appreciated by bulk secretomics. This technology will allow rapid and effective screening of functional immune cell modulation strategies designed to improve tumor-reactive T cell function.

2 Materials

2.1 Cell Culture and Single-Cell Secretome Assay

1. RPMI complete medium; RPMI medium, 10% fetal bovine serum (FBS), 0.1 mM MEM Non-Essential Amino Acid Solution, 2 mM L-glutamine, 1× penicillin/streptomycin (100 U/mL penicillin, 100 μg/mL streptomycin), 1 mM sodium pyruvate.

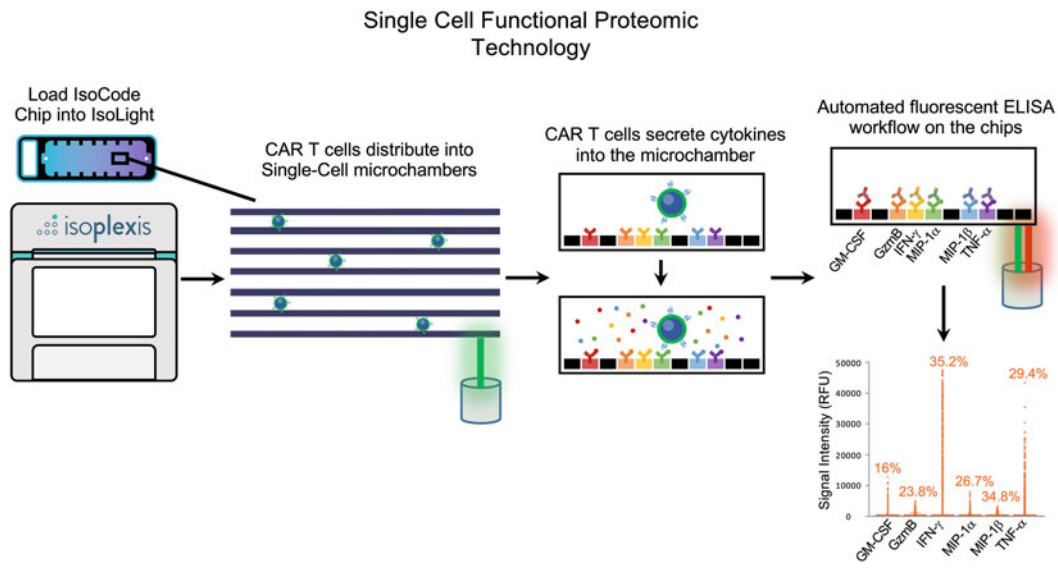

Fig. 1 Schematic representation of highly multiplexed single-cell secretomics using IsoCode chip technology. CAR T cells are loaded on an IsoCode chip, which is placed in an IsoLight instrument. The IsoLight system will automatically process the loaded IsoCode chip. Cells are separated in individual chambers within the IsoCode chip and fluorescent imaging identifies chambers with a single cell. A patterned proteomic barcode is used to capture secreted cytokines and spatial location of the proteomic barcode is used to detect cytokine signal intensity

2. AIM-V complete medium; AIM-V medium, 5% FBS, 1× penicillin/streptomycin (100 U/mL penicillin, 100 μg/mL streptomycin), 2 mM L-glutamine, 10 mM HEPES.
3. Human IL-2.
4. Phosphate-buffered saline (PBS).
5. CD19-K562 and NGFR-K562 cells.
6. aCD19-CAR T cells.
7. IsoCode chips – single-cell secretome ISOCODE-1001-4 (IsoPlexis).
8. Single-Cell Adaptive Immune panel-4 Human PANEL-1001-4 (IsoPlexis).
9. Optional: Stain Cell Membrane STAIN-1001-1 (IsoPlexis).
10. Dimethyl sulfoxide (DMSO).
11. Bovine serum albumin (BSA).
12. Target cell depletion kit (CD235a-specific microbeads, Miltenyi Biotec or Invitrogen).
13. CD8-AF647 anti-human CD8 antibody (IsoPlexis).
14. LS columns (Miltenyi Biotec).

15. MACS Separator.
16. Neubauer counting chamber.
17. Trypan blue.

2.2 Dead Cell Removal

1. Dead cell removal kit (Miltenyi Biotec).
2. Ficoll.

2.3 Intracellular Cytokine Staining

1. Monensin.
2. Brefeldin A.
3. Fixation/permeabilization kit (BD Biosciences).
4. Fluorescent-dye conjugated antibodies specific for cytokine detection by flow cytometry.

2.4 Equipment and Consumables

1. T25 and T75 cell culture flasks.
2. 24-well plate.
3. 96-well round-bottom cell culture plate.
4. 50-mL conical tubes.
5. 15-mL conical tubes.
6. Pipettes.
7. IsoLight.
8. Centrifuge.
9. Laminar flow hood.
10. Cell culture incubator.
11. Water bath.
12. Flow cytometer.

3 Methods

An overview of the protocol is provided in Fig. 2. Target tumor cell lines should be recovered 7 days before co-cultivation assay. A CD19 antigen-expressing cell line is necessary for antigen-specific stimulation of CD19-specific CAR T cells, while any CD19-negative cell line can be used as a negative control. Here, we describe the assay employing K562 cells (chronic myeloid leukemia from ATCC) that have been genetically modified to express the CD19 antigen [40]. In addition, as a negative control we use K562 cells genetically modified to express truncated low-affinity nerve growth factor (NGFR-K562) [40].

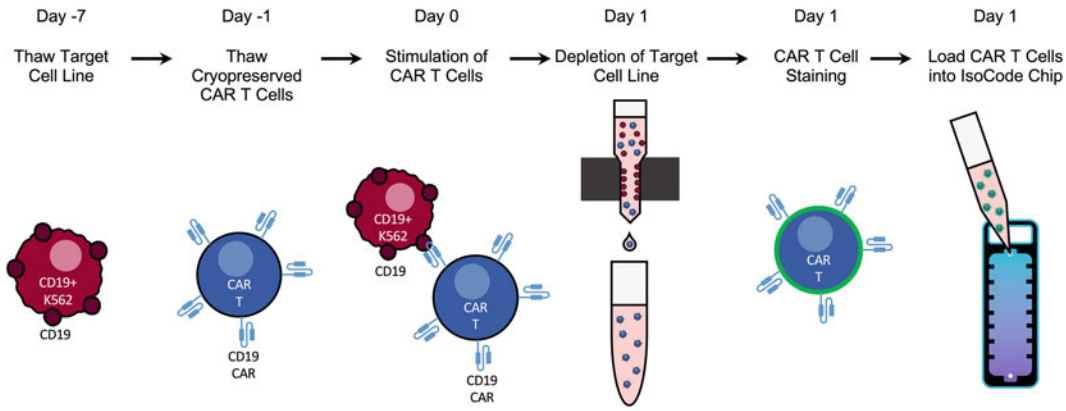

Fig. 2 Highly multiplexed single-cell secretomics protocol overview. Cryopreserved target cells are thawed 7 days before being used for stimulation of CAR T cells. Cryopreserved CAR T cells are thawed one day before their stimulation and cultivated overnight in AIM-V complete medium supplemented with 200 IU/mL IL-2. $CD8^+$ CAR T cells are stimulated by co-cultivation with a target cell line in a 1:1 ratio. After 16–18 h of incubation at 37 °C, 5% CO2, remaining tumor cells are depleted by CD235a-specific magnetic-bead separation. $CD8^+$ T cells are stained with a CD8-specific antibody (e.g., anti-CD8-AF647) and loaded on an IsoCode chip

3.1 Cultivation of Target and Nontarget Cell Lines

1. Cryopreserved target tumor cell lines should be thawed at least 7 days before stimulation of CAR T cells. To ensure enough viable cells for CAR T cell stimulation, we recommend that each cryopreserved vial contains at least 2×10^6 cells.
2. For each cell line obtain one conical 50-mL tube and label it with the cell line name.
3. Add 10 mL of RPMI complete medium to both 50-mL conical tubes.
4. Close the lids of 50-mL tubes and place the tubes in a water bath at 37 °C for 10 min.
5. Take a vial containing the cryopreserved cell line from liquid nitrogen and place it shortly in the water bath until the cell suspension is almost completely thawed.
6. Transfer cells from the cryogenic vials to the appropriate, previously labeled 50-mL tube containing RPMI complete medium, by pipetting them slowly.
7. Close lids of conical tubes and centrifuge cells at 300 × g for 10 min.
8. Discard supernatants and resuspend cell pellets in 1 mL RPMI complete medium and count cells.
9. Seed cells at a concentration of 0.5×10^6 cells/mL in T25 flask in RPMI complete medium.
10. Replace medium every 2–3 days and maintain concentration between 0.1×10^6 cells/mL and 10^6 cells/mL. Use T25 flask for up to 20 mL and T75 for up to 50 mL total volume.

3.2 Thawing Cryopreserved CAR T Cells

1. Optimally, at least 5×10^6 CAR T cells should be thawed one day before their stimulation with target T cells.
2. Please note that for optimal comparison of CAR T cell polyfunctionality, samples should contain similar frequencies of CAR T cells. In case of uneven CAR transduction rates, enrich CAR-positive T cells using magnetic-bead or flow cytometry-based sorting. Do not use antibodies or CAR detection reagents interfering with the antigen binding site of the CAR.
3. Obtain one conical 50-mL tube for each CAR T cell type, label it with CAR T cell name, and fill it with 10 mL RPMI complete medium.
4. Take a vial of each cryopreserved CAR T cell type and shortly place it in a water bath at 37 °C until cells are almost completely thawed.
5. Transfer cells to the appropriate, previously labeled conical 50-mL tube.
6. Centrifuge cells at 300 × g, 10 min at room temperature.
7. Remove supernatant and resuspend cells in AIM-V complete medium.
8. Count cells by diluting a small aliquot in trypan blue.
9. Seed cells in a 24-well plate (2 mL/well) at a concentration of 3×10^6 cells/mL in AIM-V complete medium supplemented with 200 IU/mL IL-2.

3.3 Antigen-Specific Stimulation of CD19-Specific CAR CD8$^+$ T Cell

1. Obtain two 50-mL conical tubes for CD19-K562 and NGFR-K562 cell lines and one for each CAR T cell type that will be tested and label them.
2. Take CAR CD8$^+$ T cells and target cells out of the incubator, resuspend them, and transfer them to the previously labeled 50-mL conical tube (*see* **Note 1** for bulk CAR T cells).
3. Centrifuge cells at 300 × g for 10 min.
4. Resuspend each cell pellet in 1 mL AIM-V complete medium.
5. Take a small aliquot of each cell suspension, dilute each one of them in trypan blue, and count the cells and determine cell viability (*see* **Note 2**).
6. Resuspend CD19-K562, NGFR-K562, and CD19-specific CAR CD8$^+$ T cells at a concentration of 2×10^6 cells/mL in AIM-V complete medium. Please note that stimulation of cells is performed in the absence of IL-2.
7. Take a 96-well round-bottom plate and label one part with NGFR-K562 + CAR CD8$^+$ T and the other part with CD19-K562 + CAR CD8$^+$ T. We recommend five wells for each condition.

8. Pipette 100 μL of the target cell suspension to the previously labeled wells of 96-well round-bottom plate. There should be 2×10^5 T cells/well.
9. Pipette 100 μL of CD19-specific CAR $CD8^+$ T cells in each well containing K562 cells.
10. Keep cells in the incubator at 37 °C, 5% CO2 for 13–20 h (*see* **Note 3**).

3.4 Target Cell Depletion

1. After appropriate incubation time (13–20 h; *see* **Note 3**), target cells should be depleted.
2. Depletion of CD19-K562 and NGFR-K562 can be achieved by targeting CD235a antigen with CD235a-specific microbeads (Miltenyi Biotec or Invitrogen) by following manufacturer's instructions.
3. After target cell depletion, centrifuge T cells (CD235a-negative fraction) at 300 × g for 10 min.
4. Remove supernatant, resuspend cell pellet with 1 mL PBS, and transfer cells to a 1.5-mL Eppendorf tube.
5. Proceed with membrane staining.

3.5 Chip Thawing

1. At least 4 IsoCode chips have to be loaded to start the assay.
2. Take the appropriate number of IsoCode chips out of −20 °C and keep them at room temperature until loading. Chips should be thawed 30–60 min before their loading.
3. To avoid contamination of chips it is important to keep the chips closed in the original foil during thawing.

3.6 Membrane Staining (Optional)

Membrane staining is unspecific and it stains all cells in the sample. The stain should be prepared by dilution in DMSO and PBS just before staining.

1. Thaw membrane stain at room temperature.
2. Briefly spin down the tube containing the membrane stain.
3. Add 20 μL DMSO to the vial containing the membrane stain and resuspend it with a pipette.
4. Further, dilute membrane stain in PBS (1:500 dilution).
5. Add 100 μL membrane stain for each 1×10^6 CAR $CD8^+$ T cells.
6. Incubate at 37 °C in the dark for 10 min.
7. After incubation resuspend cells and add five times the volume of complete medium.
8. Incubate for an additional 10 min at 37 °C in the dark.
9. Centrifuge cells for 10 min at 300 × g.

10. Discard the supernatant.
11. Resuspend cells in PBS supplemented with 0.5% BSA and continue with CD8 T cell staining.

3.7 CD8-AF647 Staining

1. Resuspend up to 10^6 cells in 20 μL PBS supplemented with 0.5% BSA.
2. Add 2 μL CD8-AF647 antibody.
3. Incubate in the dark at room temperature for 20 min.
4. Wash with 1 mL PBS supplemented with 0.5% BSA.
5. Optional: Take an aliquot for quality control on flow cytometer.
6. Centrifuge cells for 10 min at 300 × g.
7. Remove supernatant and resuspend cell pellet with AIM-V complete medium.

3.8 Chip Loading

1. Resuspend stained cells in AIM-V complete medium at a concentration of 10^6 cells/mL.
2. Load 30,000 cells on a chip, by pipetting 30 μL of cell suspension in the loading port of the chip. The pipette tip should slightly touch the bottom of the loading port. When pipetting, eject cell suspension only to the first stop of the pipette to avoid creating bubbles.

3.9 Loading Reagent Tubes on IsoLight

1. Briefly spin down cocktail A and cocktail B vials.
2. Add the appropriate volume (indicated on a cocktail vial) of cocktail A to the cocktail A mixing tube. Similarly, add the appropriate volume (indicated on a cocktail vial) of cocktail B to the cocktail B mixing tube.
3. To load cocktail A and B tubes, as well as reagents 1–8, open the lid of each tube, attach the sipper on IsoLight, and attach each tube by screwing the tube into the labeled position. Each tube should contain one sipper. Be careful not to cross-contaminate reagents. Make sure to use clean gloves and change them if necessary.

3.10 Run the Assay on IsoLight

1. Check CO_2 level, last clean, and the level of waste in the waste bottle.
2. Set data upload connection prior to starting the run, or alternatively, export data files after completing the run.
3. Start the program by pressing *Single-Cell Secretome* icon. Choose *Single Cell Adaptive Immune* and follow the instructions.
4. Before placing the loaded IsoCode chips in the chip compartment of IsoLight, ensure that the cell suspension entered the

chip. The loading port should not contain any liquid, and when looking at the bottom part of the chip, it should be visible that the liquid is covering approximately one-third of the chip surface.

5. Remove the blue tape from the bottom of the chip just before placing it in the IsoLight chip tray. Always place chips in the middle part of the chip tray. For example, if using four chips, two positions on the left and two positions on the right should be empty. Never leave an empty space between two chips.

3.11 Data Analysis

Once the run and data upload have been completed, remove the data upload device.

1. Launch IsoSpeak software and click on *New Project*.
2. Enter the preferred project title and select the *SC Adaptive Immune Human* application.
3. Save the project and in the IsoSpeak software click on *PROJECT INFO*.
4. Click on *IMPORT* and choose *Instrument data*.
5. Upload experiments by selecting chip IDs that should be analyzed.
6. Once the instrument data have been loaded, enter details of the project.
7. If cells were stained with the cell membrane stain, under *Cell Type* choose CD3 Violet (although the membrane stain is unspecific and will additionally stain any undepleted target cell, too).
8. Choose CD8 (red) *Cell Type* for cells stained with AF647 anti-human CD8 antibody.
9. Under *Stimulants* enter the target cell line that was used for each chip.
10. Enter information on analyst, donor ID, donor groups, viability of cells, and transduction rate.
11. Click on *DATA PROCESSING*, select all experiments, and click on *Analyze*. The analysis is completely automated and the software gives information on the progress of analysis.
12. Once the analysis is completed, the quality cells column shows the number of cells that were analyzed in each chip. The software will exclude any chamber containing more than one cell. The IsoQ score shows the quality of the analyzed data.
13. The IsoSpeak software generates tables with raw data showing signal intensity and frequency of detected cytokines, percentage of polyfunctional cells, polyfunctional strength index, and polyfunctional groups. In addition, graphs showing secretion

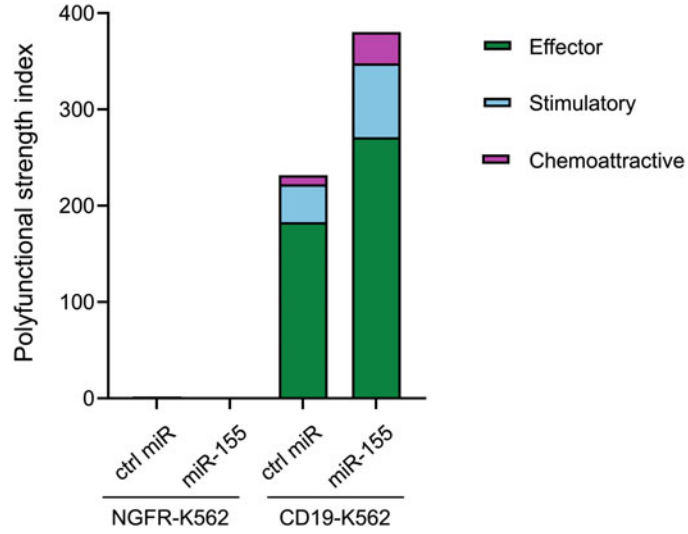

Fig. 3 Polyfunctional strength index (PSI) visualizes all single-cell, multidimensional cytokine secretions from a sample into a single index. Representative differences in PSI in CD19-specific CAR $CD8^+$ T cells overexpressing miR-155 (94.7% transduced cells) or Ctrl miR (94% transduced cells). PSI is obtained by multiplying the percentage of polyfunctional cells by the mean fluorescence intensity (MFI) of the proteins secreted by the polyfunctional cells

frequency, signal intensity, polyfunctionality, and polyfunctional strength index (PSI, Fig. 3) are automatically created. Functional heat maps (*see* Fig. 4a; *see* **Note 4**), polyfunctional activation topology PCA, and t-SNEs are also automatically generated to display multidimensional data in easy-to-understand visualizations.

14. For each graph, it is possible to apply different filters (i.e., donors, donor groups, markers), graph options, and experiment selection. If cells have been stained with both cell membrane stain and CD8-AF674, only $CD3^+CD8^+$ should be selected.
15. The graphs and raw data can show all proteins or only significantly secreted proteins. The option *Secreted* will exclude signal intensities lower than the threshold of 3 standard deviations over the background signal, cytokines secreted by less than 2% of cells or less than 20 cells. For interpretation of data obtained with miR-155 overexpressing CD19-specific CAR T cells, please *see* **Note 4**.

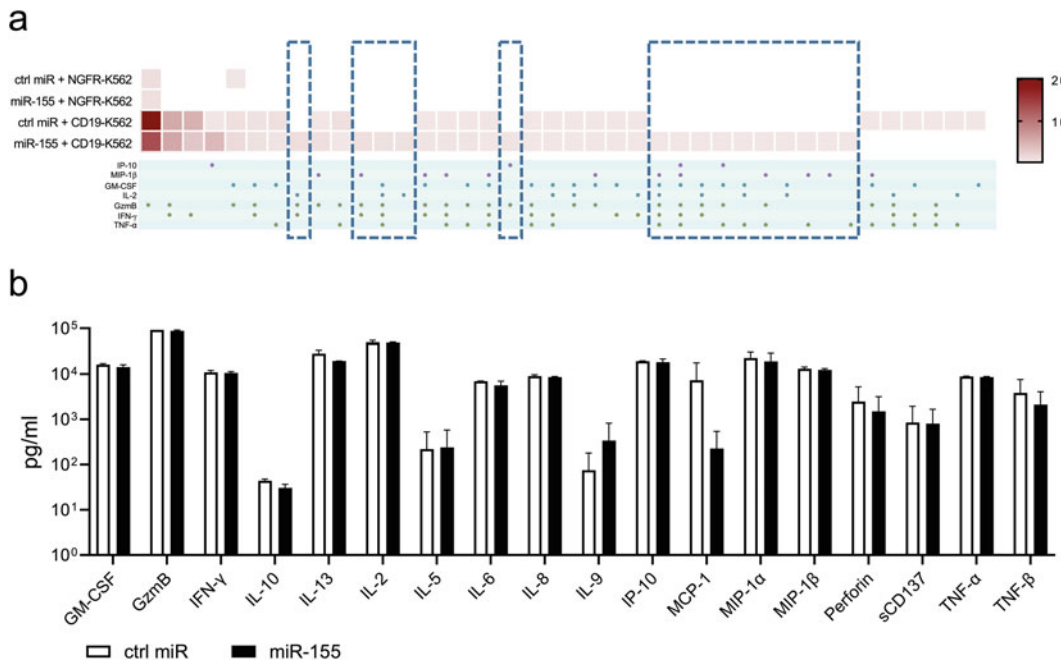

Fig. 4 Highly multiplexed single-cell but not bulk secretomics reveals enhanced polyfunctionality in CAR T cells overexpressing miR-155. (**a**) Single-cell secretome polyfunctionality heat map showing individual cell subsets of CD19-specific CAR CD8$^+$ T cells overexpressing miR-155 or Ctrl miR that simultaneously produce different combinations of cytokines. Data are shown after 16-h co-culture with CD19-K562 cells. Dashed rectangles highlight a unique polyfunctionality profile of CD19-specific CAR CD8$^+$ T cells overexpressing miR-155 from a representative healthy donor. All experiments were conducted using CD8$^+$ T cells with CD19-specific CAR transduction rates >90%. (**b**) Bulk cytokine concentration in supernatants of CD19-specific CAR CD8$^+$ T cells overexpressing miR-155 or Ctrl miR after 16-h co-culture with CD19-K562 cells. Cytokine concentration was determined by CodePlex technology. Bars show mean cytokine concentrations of antigen-stimulated CD19-specific CAR CD8$^+$ T cells from two healthy donors. Error bars are the standard error of the mean (SEM)

4 Notes

1. CAR CD4$^+$ T or CAR CD8$^+$ T cells should be loaded separately on individual IsoCode chips. These two CAR T cell populations can be enriched from bulk CAR T cells by positive selection using CD4 or CD8 MicroBeads (Miltenyi Biotec). Alternatively, a CD8- or CD4-negative enrichment kit can be used (Miltenyi Biotec or STEMCELL Technologies). This step should be performed before CAR T cell stimulation.
2. If the viability of cells is lower than 80%, remove dead cells by Ficoll gradient centrifugation or by Dead Cell Removal Kit (Miltenyi Biotec). To remove dead cells by Ficoll gradient centrifugation, centrifuge the cell suspension at 300 × g for 10 min, remove the supernatant, and resuspend the cell pellet

in 5 mL AIM-V complete medium. Add 5 mL Ficoll to a 15-mL conical-bottom tube. Carefully layer the cell suspension on top of the Ficoll. Centrifuge cells at 700 × g for 20 min at room temperature without a break. Carefully collect viable cells from the layer between Ficoll and AIM-V medium, and transfer them to a new 15-mL tube. Resuspend cells in 1 mL AIM-V complete medium. Count cells and determine cell viability.

3. To determine the optimal incubation time of CD19-specific CAR T cells and target cells, it is important to detect cytokine production in an independent experiment. This can be done by intracellular cytokine staining or by measuring bulk cytokine concentration in the cell culture supernatant. For both of these two approaches, co-cultivation experiment (*see* Methods, 3.3 Antigen-specific stimulation of CAR T cells) should be performed for 6 h, 12 h, and 18 h. After the co-cultivation time is over, target cells should be depleted (see Methods, 3.4 Target cell depletion) and cytokine production should be evaluated either by intracellular cytokine staining or by measuring bulk cytokines.

   For intracellular cytokine staining, add monensin (2 μM) and brefeldin A (1 μg/mL) to the CD19-specific CAR T cells and continue incubation at a concentration of 10^6 cells/mL in AIM-V complete medium for additional 13 h. After 13 h, permeabilize cells with saponin-containing buffer and perform intracellular cytokine staining (e.g., IFNγ, TNFα, IL-2). Perform flow cytometry and determine the co-cultivation duration time that results in the highest cytokine staining. In our example (*see* Fig. 5), the percentage of TNFα- and IL-2-positive cells did not change much with stimulation time. However, according to IFNγ-positive cells, optimal co-incubation time was anywhere between 12 and 18 h.

   Alternatively, for bulk cytokines in the supernatant, incubate CD19-specific CAR T cells depleted of target cells at a concentration of 10^6 cells/mL in AIM-V complete medium at 37 °C, 5% CO2 for 13 h. Collect supernatants and measure cytokine concentration by any available assay such as multiplex ELISA or IsoLight CodePlex secretome assay.

4. In our example, CD19-specific T cells overexpressing miR-155 comprise higher fractions of cell subpopulations displaying unique polyfunctional profiles compared to CD19-specific CAR T cells engineered with a Ctrl miR (*see* Fig. 4a). Notably, no significant differences in total cytokine production nor polyfunctionality are observed when cytokine release is evaluated by bulk secretomic technology (*see* Fig. 4b). Additionally, the PSI can be used to reduce the multidimensionality of the

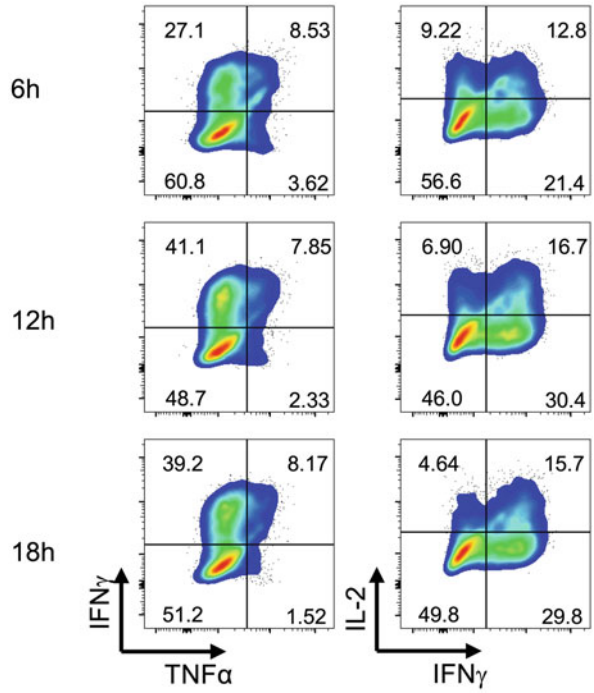

Fig. 5 Optimization of CAR T cell/target cell co-culture timing by intracellular cytokine staining (ICS). ICS of $CD8^+$ CD19-specific CAR T cells stimulated with CD19-K562 cells for 6 h, 12 h, or 18 h. Data are shown from a representative healthy donor

dataset into a single metric that reflects overall polyfunctional cytokine secretion strength. PSI is computed by multiplying the percentage of polyfunctional cells by the mean fluorescence intensity (MFI) of the secreted cytokines.

Conflicting Interests

L.G. has consulting agreements with Lyell Immunopharma, Instil Bio, and Advaxis. L.G. is on the scientific advisory board of Poseida Therapeutics and Kiromic and a stockholder of Poseida Therapeutics. E.H. and J.Z. are employed by and have equity ownership in IsoPlexis.

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Chapter 12

Genome Editing in CAR-T Cells Using CRISPR/Cas9 Technology

Irene Andreu-Saumell, Alba Rodriguez-Garcia, and Sonia Guedan

Abstract

CAR-T cell therapy is revolutionizing the treatment of hematologic malignancies. However, there are still many challenges ahead before CAR-T cells can be used effectively to treat solid tumors and certain hematologic cancers, such as T-cell malignancies. Next-generation CAR-T cells containing further genetic modifications are being developed to overcome some of the current limitations of this therapy. In this regard, genome editing is being explored to knock out or knock in genes with the goal of enhancing CAR-T cell efficacy or increasing access. In this chapter, we describe in detail a protocol to knock out genes on CAR-T cells using CRISPR–Cas9 technology. Among various gene editing protocols, due to its simplicity, versatility, and reduced toxicity, we focused on the electroporation of ribonucleoprotein complexes containing the Cas9 protein together with sgRNA. All together, these protocols allow for the design of the knockout strategy, CAR-T cell expansion and genome editing, and analysis of knockout efficiency.

Key words Genome editing, CAR-T cells, CRISPR/Cas9, T-cell engineering

1 Introduction

Adoptive transfer of CAR-T cells has shown tremendous promise for the treatment of cancer [1]. Treatment with autologous CAR-T cells targeting CD19 or BCMA can achieve high rates of long-term complete responses in patients with relapsed/refractory leukemia and lymphoma or multiple myeloma, respectively [2]. While the power of CAR-T cells in B-cell malignancies is truly unprecedented, the majority of patients with solid tumors or certain hematologic malignancies do not benefit yet from these therapies. Translation of CAR-T therapy to more difficult-to-treat tumors will require further genetic modifications of CAR-T cells, including elimination of genes that could diminish CAR-T cell efficacy or overexpression of genes that can drive more potent antitumor responses [3].

Irreversible silencing of protein expression can be easily achieved using genome editing tools that allow efficient knockout

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of genes of interest [4, 5]. Genome editing technology consists on the combination of engineered nucleases with sequence-specific DNA-binding domains that directs the nuclease to the target DNA cut site [6]. In the field of CAR-T cells, genome editing approaches have been used in three main applications [3]. First, the KO of components of the TCR and the HLA allow for the generation of allogeneic universal "off-the-shelf" CAR-T cells [4]. Significant efforts are under way to translate the use of universal CAR-T cells into the clinics, with the first clinical trials already reporting feasibility, safety, and efficacy [7, 8]. Second, genome editing approaches have been used to generate CAR-T cell products resistant to fratricide for the treatment of T-cell malignancies [9]. In this regard, CAR-T cells targeting CD7 with disrupted CD7 and TRAC genes are already being tested in the clinic [10]. Finally, strategies to silence inhibitory receptors or any other protein that can impair CAR-T cell efficacy are also being widely explored [11–13].

Long-term expression of novel or native proteins is typically achieved using retroviral and lentiviral vectors or transposon systems that can randomly integrate the gene of interest in the T-cell genome [3]. A more elegant strategy would be to knock in the gene of interest into selected endogenous loci, allowing the expression of the transgene under the natural promoter of the targeted gene. Considerable progress in the field over the past years make it now possible to knock in genes in specific loci using these same genome editing tools in combination with a donor DNA that encodes the transgene of interest [14, 15]. The first approach in the field in this regard was focused on knocking in the CAR into the endogenous TCR locus [14]. As the field expands, other transgenes (such as IL-12) are being knocked in in different loci (such as PDCD1 or CD25) [16]. While the field is poised for rapid advancement, as of now, the protocols for knock-in approaches in T cells require further optimization to increase efficiency and will not be discussed in this chapter.

Different genome editing tools have been efficiently used to install the desired genetic modifications, including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9 platform [6]. Due to its simplicity, flexibility, and effectiveness, in this chapter we will focus on the use of the CRISPR–Cas9 platform. The protocols described here allow for the obtention of genome-edited CAR-T cells with high knockout efficiencies. Methods and tools to test the efficacy and toxicity of these genome-edited CAR-T cells have been reviewed elsewhere [17, 18].

2 Materials

2.1 T-Cell Isolation

1. Fresh blood or buffy coat.
2. Lymphoprep (StemCell Technologies Catalog # 7811).
3. Phosphate-buffered solution without calcium and magnesium (PBS −/−, Invitrogen, catalog # 20012–068).
4. Fetal bovine serum (FBS) (Merck, catalog # F4135).
5. RosetteSep Human CD8+ T-cell enrichment kit/human CD4+ T-cell enrichment kit (StemCell Technologies, catalog # 15062 and 15,063).
6. R10 medium: RPMI 1640 (Merck, Catalog # R6504-10X1L) supplemented with 10% (v/v) heat-inactivated FBS, 1X GlutaMax (Thermo Fisher, catalog # 35050–061), 100 μg/mL penicillin, 100 U/mL streptomycin (Thermo Fisher, catalog # 15140122), 10 mM HEPES (Sigma, catalog # H0887-100ML), 10 ng/mL human recombinant IL-7, and 10 ng/mL human recombinant IL-15 (Miltenyi, catalog # 130–095-362 and 130–095-764).
7. Anti-CD3/CD28 magnetic beads (Invitrogen, catalog # 11132D).
8. DynaMag-2 and Dynal 15-mL magnet (Invitrogen, catalog # 12321D and 12301D).
9. Hemocytometer ([BRAND™]_VWR, catalog # BE718605).
10. Trypan blue solution (Sigma, catalog # T8154).

2.2 Generation of CRISPR/Cas9-Edited CAR-T Cells

11. CAR-expressing lentivirus vector stock.
12. Multiparameter flow cytometer (e.g., BD FACSCanto II).
13. FlowJo software.
14. Chemically modified sgRNA (CRISPRevolution sgRNA EZ Kit, Synthego).
15. Neon™ Transfection System 100 μL Kit (Thermo Fisher, catalog # MPK10096).
16. Neon™ Transfection System Pipette (Thermo Fisher, catalog # MPP100).
17. Neon™ Transfection System Pipette Station (Thermo Fisher, catalog # MPS100).
18. Neon™ Transfection System (Thermo Fisher, catalog # MPK5000).
19. TrueCut™ Cas9 Protein v2 (Thermo Fisher, catalog # A36499).
20. Deionized water (Thermo Fisher, catalog # 15230089).

Table 1
List of antibodies to analyze T-cell purity, CD4/CD8 T-cell ratio, CAR, and PD-1 expression during primary expansion by flow cytometry

Target	Fluorochrome	Catalog #	Company
CD3	PE	12–0037-42	Thermo fisher
CD4	FITC	557,695	BD bioscience
CD8	APC	17–0086-42	Thermo fisher
CAR (murine scFv)	Biotin goat anti-mouse IgG	115–065-072	Jackson ImmunoResearch
CAR (human or humanized scFv)	Biotin goat anti-human IgG	109–066-006	Jackson ImmunoResearch
Streptavidin	PE	12–4317-87	Thermo fisher
PD-1	PeCy7	561,272	BD bioscience

2.3 Analysis of Knockout Efficiency

21. DNA extraction kit (e.g., DNeasy Blood & Tissue Kit, Qiagen, catalog # 69504).
22. High-fidelity DNA polymerase, buffer, and dNTPs (e.g., Phusion ® High-Fidelity DNA Polymerase, NEB, catalog # M0530S).
23. 10 μM forward and reverse primers (IDT).
24. PCR cleanup kit (e.g. PCR PureLink™, Thermo Fisher, catalog # K310001).
25. Nuclease-free water (Thermo Fisher, catalog # AM9906).
26. Standard 1% agarose gel.
27. Tris–acetate EDTA (TAE) buffer (Bio Rad, catalog # 1610743).
28. DNA ladder 1 kb (WERFEN ESPAÑA S.A.U, catalog # 174N3232S).
29. DNA loading dye (Thermo Fisher, catalog # R0611).
30. Gel electrophoresis equipment.
31. Thermocycler.
32. Tris-Buffered Saline (TBS) (Bio Rad, catalog # 1706435).
33. Tween-20 (PanReac-AppliChem, catalog # A4974,0250).
34. GraphPad software (Table 1).

3 Methods

In this protocol, CRISPR/Cas9 technology will be used to genetically edit CAR-T cells. This methodology relies on two components: a single-guide RNA (sgRNA) which will bind to the targeted

DNA sequence and the endonuclease Cas9 which will induce DNA double-strand breaks (DSBs) at that specific point. Then, error-prone endogenous DNA repair mechanisms will introduce insertions or deletions (indels), potentially leading to loss-of-function mutations of the targeted gene.

Since primary T cells are hard-to-transfect cells, alternative methods are needed to deliver the required components to the cells. Methods based on the delivery of Cas9 and/or sgRNA by using either non-integrating (adenoviral) or integrating (lentiviral) viral vectors have been reported [19, 20]. However, limited transduction rates result into relatively low editing efficiencies, and multiplex editing is limited by the packaging capacity of the vector [21]. In addition, in the case of integrating vectors, the long-term expression of Cas9 and sgRNA can lead to undesirable off-target effects. Alternatives to this could be to incorporate multiple sgRNAs in the CAR lentiviral vector and deliver the Cas9 by the electroporation of protein or mRNA [22], or to deliver in vitro-transcribed sgRNA and Cas9 RNA by electroporation [5]. DNA nucleofection is another CRISPR/Cas9 delivery method by which T-cell editing has been achieved, although it is associated to high toxicity to T cells [23, 24].

In this protocol, we will genetically modify T cells by electroporation of a ribonucleoprotein (RNP), consisting of a Cas9 protein complexed with sgRNA. This method is simple and fast and has shown good efficiency on T cells as well as reduced off-target effects and toxicity as compared to the above-described methods [25]. In addition, it is a flexible platform that allows for multiplex editing [12] and it has been reported to enhance efficiencies of challenging knock-in approaches [25].

3.1 Generation of CAR-T Cells

The starting amount of T cells will depend on the intended application for the expanded cells. Taking into account that electroporation can result into 50% of T-cell death, a 10- to 30-fold increase in T-cell numbers is expected from day 0 to day 10. An example of the numbers used to start a CAR-T cell expansion when electroporating RNPs is shown in Table 2.

3.1.1 T-Cell Isolation

1. Isolate $CD4^+$ and $CD8^+$ T cells from blood or buffy coats using RosetteSep T-cell-negative selection kit following manufacturer's instructions (*see* **Note 1**).
2. Assess T-cell purity and CD4/CD8 T-cell ratio by staining the purified sample with anti-CD3, anti-CD4, and anti-CD8 antibodies for flow cytometry analysis. The T-cell purity ($CD3^+$) should be above 95%.

Table 2
Example of T-cell numbers used to start an expansion depending on the application

	Million T cells			Observations
	Day 0	Day 4 (electroporation)	Day 10	Electroporation of T cells can be performed on day 2. It is important to take into account that T cells do not double till day 3–4. Electroporating less than two million cells can result in increased T-cell death
In vitro studies	2	4	20–60	
In vivo studies	5	10	100–300	

3.1.2 T-Cell Stimulation and Transduction

1. Prepare T cells in R10 complete medium to a concentration of 1×10^6 cells/mL and aliquot the required number of cells into appropriate multi-well flat-bottom plates or flasks. Use as many wells as experimental groups. Include a control group of untransduced T cells (UTD) (*see* **Note 2**).
2. Based on a ratio of two beads to one T cell, calculate the amount of anti-CD3/CD28 magnetic beads needed (*see* **Note 3**).
3. Thoroughly resuspend the magnetic beads and transfer the calculated volume to a 1.5-mL tube placed on the DynaMag-2 magnet.
4. After 1 min, discard the beads' buffer. Remove the tube from the magnetic field and wash the beads with 1 mL of pre-warmed R10 medium. Repeat the washing step twice.
5. After the last wash, resuspend the beads in a small volume of R10 medium (i.e., 50 μL of media per million beads).
6. Transfer the beads to the corresponding wells containing the T cells and mix.
7. Twenty-four hours later, transduce the activated T cells with the CAR-encoding lentivirus at a multiplicity of infection (MOI) of 5 (*see* **Note 4**). Simply add the volume of virus needed to achieve the corresponding MOI and mix gently.
8. Feed the cells with one volume of R10 complete medium on the third day after stimulation.

3.2 Gene Editing by CRISPR/Cas9 Technology

For most targets, gene editing can be done indistinctly two to four days after stimulation, when T cells have received sufficient activation from the CD3/CD28 beads and are actively proliferating. However, for certain targets, genome editing might be required at earlier timepoints. This can apply, for instance, to CAR-T cells targeting antigens that are expressed on T cells, in order to avoid co-expression of the CAR and the targeted antigen and to prevent fratricide (i.e., CD7). In this scenario, the knockout should be

Table 3
Suggested experimental CRISPR/Cas9 controls

Control	Description	Purpose
Positive control	CAR-T cells are electroporated with Cas9 complexed with sgRNAs that have demonstrated high editing efficiency (i.e., sgRNA for TRAC)	Ensures that all reagents, protocol, and equipment are functioning at optimal conditions This control might be used when optimizing a protocol or when trying a sgRNA for the first time
Negative control: Non-electroporated T cells	T cells are not electroporated and cultured in the absence of Cas9 and sgRNA	This control determines cell growth at basal conditions
Mock control: Electroporated T cells	CAR-T cells are electroporated with Cas9 complexed with a nontargeting sgRNA, a sgRNA targeting a genomic safe harbour (i.e., AAVS1 or Rosa26) or an intron, or with no sgRNA.	It controls for toxicity from RNP (or Cas9), cell death from electroporation, or possible viability issues associated with editing the specific gene of interest. Ensures that the observed phenotype is due to the specific editing and not to the transfection process No indels are expected to occur at any genome location
	CAR-T cells are electroporated with no Cas9 or sgRNA	It might be used to avoid costs associated with the electroporation of RNP or Cas9. It is similar to the above control except it does not control for RNP and/or Cas9 toxicity It is highly recommended to have a mock control in all studies

achieved before the CAR is expressed in the T-cell membrane (*see* **Note 5** for protocol variations). A list of suggested experimental CRISPR/Cas9 controls to include as part of the expansion protocol is shown in Table 3.

3.2.1 Debeading

Prior to electroporation, beads must be removed from the cell culture and electroporation of RNPs is performed on the same day.

1. Count T cells using trypan blue exclusion or an automated cell counter (*see* **Note 6**).
2. Calculate the volume of R10 medium needed to adjust the cell concentration to 1×10^6 cells/mL.
3. Place two uncapped 15-mL tubes per sample on the 15-mL Dynal magnet.

4. Mix the cells thoroughly in their own medium to free the cells off the beads.
5. Transfer the sample into the first falcon tube. After 1 min, the beads should accumulate on the magnet-side walls.
6. Without disturbing the beads, carefully transfer the sample to the second tube for another minute and then transfer the sample into the corresponding well/flask (refer to **step 2** from Subheading 3.2.4 to determine the container). This step is required to assure that all beads have been eliminated from the T-cell culture.
7. Following the sequential order of transfer, use the previously calculated volume of medium in **step 2** to wash the beads after each transfer and feed the cells with the washing medium. This step is necessary to collect T cells that remain bound to the beads.
8. Leave the cells resting in a humidified 37 °C, 5% CO2 incubator for 2–4 h before the electroporation step.

3.2.2 Single-Guide RNA (sgRNA) Design

For this protocol, the CRISPR Design Tool from Synthego (https://www.synthego.com/products/bioinformatics/crispr-design-tool) was used to design the sgRNA (*see* **Note 7**). This tool uses a sequential algorithm to rank candidate guide RNA sequences that have a high chance of knocking out the gene of interest while minimizing off-target effects. To be suggested as candidates, guides need to accomplish the following features: (I) target a common exon in the primary transcript, (II) target an early region of the gene, (III) have an on-target score of >0.5 based on the Azimuth 2.0 model, and (IV) have no off-target sites within the same genome that have 0, 1, or 2 mismatches compared to the guide RNA sequence. Chemically modified sgRNAs which have shown to provide superior editing in most cell types (including T cells) were used [26]. Of note, it would be recommendable to test various sgRNA for the same target gene in order to screen for the best candidate. Screening of sgRNAs can be alternatively performed in Jurkat cells in order to reduce costs associated to T-cell activation, transduction, and expansion, as well as to save time.

3.2.3 T-Cell Preparation

1. Centrifuge T cells at 300 × *g* for 7 min and remove the supernatant. **Important!** Keep the conditioned media (supernatant) to resuspend T cells after electroporation, as it contains cytokines and factors secreted by T cells that are required for proper T-cell expansion.
2. Add 10–20 mL of PBS (no calcium, no magnesium) and centrifuge T cells at 300 × *g* for 7 min.
3. Resuspend the T cells in Resuspension Buffer R at the desired concentration in order to have 2–3 × 10^6 cells per reaction in a

volume of 50 μL and place the cells on ice for no longer than 20 min, as this will reduce cell viability and transfection efficacy (*see* **Note 8**). **Important!** The number of cells per reaction could be increased up to 8–10 × 10^6 cells, although this might reduce KO efficiency (around 50% depending on the target locus). This lower efficiency may be desired in specific cases (e.g., to evaluate if the elimination of the gene of interest generates an enrichment of the knockout T-cell population). However, if higher editing efficiencies are desired (>80%), T cells should be electroporated in rounds of 2–5 × 10^6 cells.

3.2.4 Preparation of RNP Complexes

1. During the T-cell centrifugation step, prepare RNP complexes. Mix 10 μg of Cas9 protein (2 μL of TrueCut™ Cas9 Protein v2) and 2.5 μg of sgRNA (~2 μL of 100 μM – 100 pmol/μL sgRNA) in a final volume of 50 μL of Resuspension Buffer R per reaction. The molar ratio Cas9/gRNA is 1:3.3. Mix well gently (*see* **Note 9**).
2. Incubate the Cas9/gRNA complex at room temperature (RT, 15 to 25 °C) for 5–20 min.
3. Add the Cas9/gRNA complex (from **step 5**) to the T cells (from **step 6**) and mix well.

3.2.5 Electroporation of RNP Complexes

1. Pipette 100 μL of the T cells mixed with Cas9/gRNA complexes into the Neon™ 100-μL tip. **Important!** Avoid creating air bubbles while loading the electroporation tip, as this will result in lowered or failed transfection. If air bubbles are noticed in the tip, the sample must be returned to its tube and carefully pitetted again.
2. Use program #24 (1600 V/10 ms/3 pulses) for electroporation.
3. Immediately transfer the electroporated cells into the appropriate vessel containing the needed volume of conditioned medium to adjust CAR-T cell concentration to 1.5 × 10^6 cells/ml and transfer the plate to a humidified 37 °C, 5% CO2 incubator (*see* **Note 10**). After 48 h, proceed with post-editing CAR-T cell expansion.

3.2.6 Postediting CAR-T Cell Expansion

During the logarithmic phase of T-cell expansion (days 6–9), T cells must be counted and fed daily with fresh medium in order to prevent the T cell concentration get above 2 × 10^6 cells/mL. Maintain T cells in culture until they rest down (as determined by both decreased growth kinetics and cell size). Following this protocol, this will typically happens around day 10–12 after activation, when T cells can be cryopreserved or used for functional assays.

1. Count the cells using trypan blue exclusion or an automated cell counter.

Table 4
Recommended plates and flask sizes for the T-cell expansion

N	Well plate/flask type
2 to 5	24-well plate
5 to 8	12-well plate
8 to 20	6-well plate
20 to 40	T25 flask–horizontal
40 to 112.5	T75 flask–horizontal
>112.5	T150 flask–horizontal

The "N" column specifies the range of values for the parameter N. The "Well plate/flask type" column provides the corresponding type of container for each size range. The term "horizontal" indicates the orientation of the flask

2. Transfer the cells to the appropriated well or flask based on the formula and Table 4 below:

$$N = \frac{Total\ cell\ number}{3.5\ x\ 10^5}$$

3. Add fresh R10 medium to adjust the cell concentration to 0.8×10^6 cells/mL. To minimize cell loss during well/flask transfers, use the feeding medium to wash the well/flask.
4. Use 1×10^5 to 2×10^5 cells to assess CAR expression on $CD4^+$ and $CD8^+$ T cells at the end of primary expansion (day 8–10). CARs can be stained by using either conjugated target recombinant proteins or anti–mouse/human IgG (depending on the CAR's ScFv origin). Anti-CD4 and anti-CD8 antibodies may also be included, and if the protein being knocked out is expressed on the cell surface, specific antibodies might be also included to assess KO efficiency as described below (*see* Subheading 3.3.2). Analyze by flow cytometry.
5. **(Optional)** If genome editing is on a surface molecule (i.e., CD3, T-cell receptor components, or components of the MHC-I complex such as β2m), edited CAR-T cells could be enriched by negative selection (e.g., by using magnetic microbeads) before cryopreservation or functional experiments.
6. Keep at least 1×10^6 cells per group to quantify editing efficiency (*see* Subheading 3.3.1).
7. Cryopreserve T cells by day 10 or use them fresh by day 10 to 12. $CD4^+$ and $CD8^+$ T cells can be cryopreserved and/or tested separately or can also be mixed at a 1:1 ratio for cryopreservation and/or further characterization.

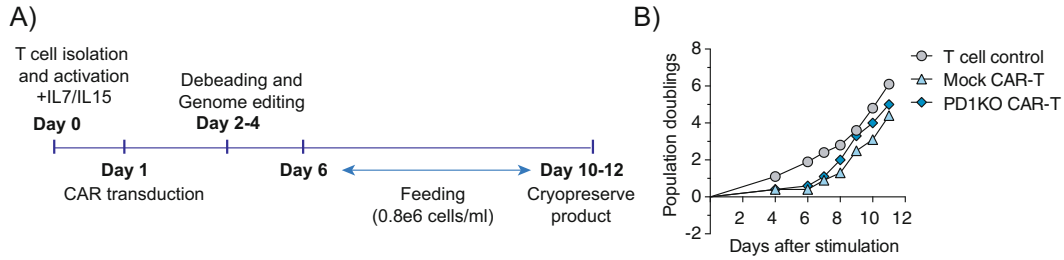

Fig. 1 Ex vivo expansion of gene-edited CAR-T cells. (**a**) Schematic representation of T-cell expansion and CRISPR/Cas9 editing protocol in primary human CAR-T cells. (**b**) Population doublings in genome-edited CAR-T cells during primary T-cell expansion

8. Calculate population doubling: Use the total cell numbers obtained from day 0 to the end of T-cell primary expansion (day N) to calculate the population doubling relative to the number of cells stimulated on day 0, using the following formula:

$$\text{Pop.Doub.} = \left(\log_2 \text{Total Cells}_{\text{DayN}}\right) - \left(\log_2 \text{Total Cells}_{\text{Day0}}\right)$$

T cells should double approximately 4–5 times from day 0 to day 10. Population doublings below 4 at day 10 are unusual and may indicate a problem during T-cell expansion. Representative T-cell expansion data after gene editing is shown on Fig. 1b.

3.3 Analysis of Knockout Efficiency

Assessment of editing efficiency is a critical step and can be done at different timepoints during T-cell expansion (i.e., at day 6, 8, and 10) to assess if the knockout provides a proliferative advantage to the cells, resulting in an enrichment of the gene-edited population (or the opposite). Editing efficiency can be determined at DNA or protein level.

For assessing editing efficiency at the DNA level, genomic DNA must be extracted and the target gene amplified by PCR. Then, different methodologies can be used to assess KO efficiency. Mismatch repair assays such as T7E1 or Surveyor Mismatch Cleavage rely on the activity of T7 nucleases that cleave DNA when there are mismatches, when editing has occurred. These methods are time-consuming and not very accurate and often underrepresent editing efficiency [27]. More accurate methods are those based on sequencing. Ideally, next-generation sequencing (NGS) of amplicons could be used, but elevated cost prevents its routine use. More cost-effective methods such as inference of CRISPR edits (ICE, Synthego) or tracking of indels by decomposition (TIDE) rely on Sanger sequencing to resolve indel size frequencies from edited cell populations by comparing and decomposing Sanger traces made from PCR products of targeted regions from unedited/mock and edited templates. For this protocol, we have used ICE to assess editing efficiency.

For assessing editing efficiency at the protein level, flow cytometry (surface or intracellular staining), ELISA, or western blot could be used depending on the cellular localization of the targeted protein.

3.3.1 DNA Level

1. Extract genomic DNA from at least 1×10^6 cells (from both edited and unedited/mock pool of cells) by using a commercial DNA extraction kit according to the manufacturer's specifications (e.g., DNeasy Blood & Tissue Kits, QIAGEN).
2. Use 150 ng of genomic DNA to amplify the targeted region by standard polymerase chain reaction (PCR) (i.e., Phusion ® High-Fidelity DNA Polymerase from NEB). For this protocol, the benchling software (https://benchling.com/) was used to design specific primers flanking the region including the potential cleavage site (*see* **Note 11**).
3. Purify PCR product by using a PCR purification kit (e.g., PCR PureLink™, Thermo Fisher). Use a sample of the purified product to confirm amplification by running a 1% agarose electrophoresis gel.
4. Perform Sanger sequencing of the purified amplicons from the mock and edited samples. Primers used for the amplification step might be used as long as they are at a distance enough to ensure good sequencing quality at the region containing the indels.
5. Quantify the total indel percentage (frequency of sequences that contain an insertion or deletion) and the knockout score (frameshift-inducing indels or deletions of >21 bp) by using ICE (https://ice.synthego.com/). A higher knockout score will indicate a higher likelihood of indels resulting in a functional KO of the targeted gene. R2 values indicate how well the indel distribution fits the Sanger sequence data of the edited sample. All the obtained parameters can be then graphically displayed (*see* Fig. 2).

3.3.2 Protein Level

1. For target proteins expressed at the cell surface, stain 2×10^5 T cells with fluorochrome-labeled antibodies for flow cytometry analysis and compare expression levels in mock/unedited samples versus edited cells (e.g., PD-1 and CD3, Fig. 3a and b).
2. For target proteins that are secreted (e.g., cytokines), supernatants can be analyzed by enzyme-linked immunosorbent assay (ELISA) for protein detection.
3. For target proteins expressed intracellularly, intracellular staining (ICS) protocols can be performed for flow cytometry analysis as well as protein detection by standard western blot protocols.

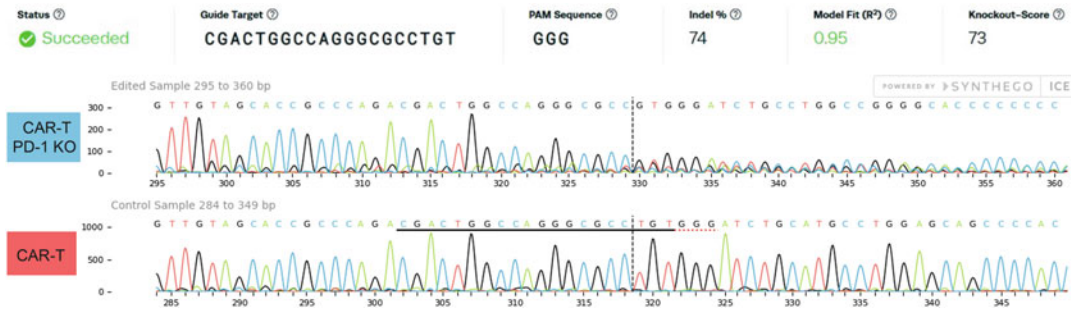

Fig. 2 Example of a Trace tab of PD-1 KO obtained using the ICE analysis tool. The traces tab edited (CAR-T PD-1 KO) and control (CAR-T, bottom) Sanger traces in the region around the guide RNA binding site (horizontal black line) and the PAM site underlined with a red dotted line. The cut site is indicated with a vertical dotted line on both traces

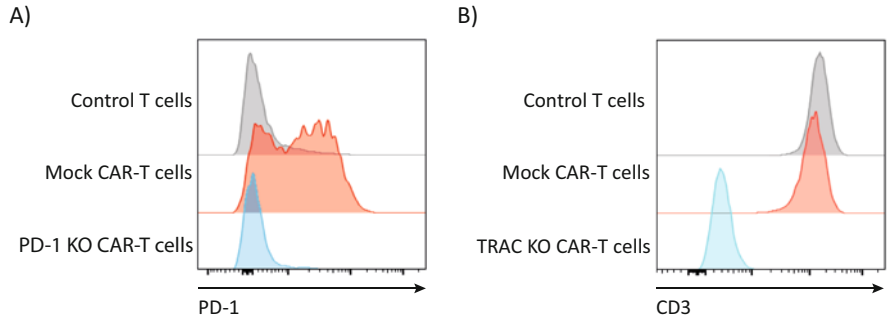

Fig. 3 (**a**) PD-1 expression was assessed by flow cytometry in CAR-T cells at day 8 of T-cell expansion. (**b**) CD3 surface expression on CAR-T cells after CRISPR/Cas9 KO of TRAC as assessed following antigen stimulation

4 Notes

1. Other technologies may be used (e.g., microbeads from Miltenyi Biotec).
2. Media supplementation with IL-7 and IL-15 until day 9 of expansion limits T-cell differentiation during T-cell culture and is reported to improve antitumor efficacy of CAR-T cells [28–30].
3. The manufacturer recommends a bead/cell ratio of 1:1. However, a ratio of 2:1 may be used to obtain a better stimulation of the cells.
4. Lentivirus should have been previously tittered in sub-T1, Jurkat cells, 293 T cells, or T cells.
5. An alternative genome editing protocol is to perform CRISPR/Cas9 on day 2 post-stimulation and to transduce T cells with the CAR lentivirus on day 3 (instead of day 1). From day 5 to 10, proceed with typical CAR-T cell expansion.

Alternatively, naïve (non-stimulated) T cells could be genome edited on day 1 after isolation. Electroporation conditions as well as IL-7/15 concentrations must be modified in this case (i.e., 2200 V 20 ms 1 pulse, 25 ng/mL IL-7/15) [31]. Then, T cells are activated on day 2, transduced with the lentiviral CAR on day 3, and expanded up to day 12.

6. Other automated cell counter with cell diameter gating options and with cell volume or diameter average display can be used (e.g., Countess, from Life Technologies).
7. Other informatic tools might be used for sgRNA design (e.g., Benchling, GeneArt CRISPR Search, and Design tool from Invitrogen, CRISPR–Cas9 guide RNA design checker from IDT, E-CRISPR, CHOP-CHOP, etc.).
8. It is recommended to prepare an extra amount of cells to avoid pipetting errors.
9. It is recommended to prepare an extra amount of RNP complexes to avoid pipetting errors.
10. Electroporation causes significant cell death (roughly 50% of the total population). Taking this into account, it is recommended to leave the T cells at a high concentration, of at least 1.5×10^6 cells/mL.
11. For best results: (I) use primers with Tm > 55 °C, (II) design primers that are 18–22 bp in length and have 45–60% GC content, (III) primers should yield amplicon lengths between 400 and 500 bp, and (IV) the potential cleavage site should not be in the center of the amplicon so the detection reaction will yield two distinct product bands that could be easily distinguished. Amplification of difficult targets might require troubleshooting and PCR optimization. For instance, a temperature gradient can be used to optimize the annealing temperature for each primer pair. Amplification of GC-rich sequences or sequences with secondary structure may be improved by the presence of additives such as DMSO or by the use of specific buffers.

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Chapter 13

Genetic Modification of Tumor-Infiltrating Lymphocytes, Peripheral T Cells, and T-Cell Model Cell Lines

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Abstract

Genetic modification of tumor-infiltrating lymphocytes (TILs) or circulating T cells has become an important avenue in cancer therapy. Here we describe a comprehensive method for establishing and expanding TIL cultures and genetically modifying them with a gene of interest (GOI) via retroviral transduction or mRNA transfection. The method includes all the important steps starting with TIL extraction from tumors through to the maintenance of the genetically modified TILs. The protocol includes instructions for retroviral transduction and mRNA transfection of circulating T cells or T-cell lines. The GOIs most commonly introduced into the target cells are chimeric antigen receptors (CARs); genetic adjuvants, such as membrane-bound interleukins; and antitumor T-cell receptors (TCRs).

Key words CD8 T cells, mRNA electroporation, Chimeric antigen receptors, Tumor-infiltrating lymphocyte, Adoptive cell therapy, Rapid expansion protocol, T cell activation, Retroviral transduction, T cell activation, Preparation of T cells

1 Introduction

Adoptive transfer of tumor-infiltrating lymphocytes (TILs), gene-modified T cells expressing antitumor T-cell receptors (TCRs), or chimeric antigen receptors (CARs) yields a high rate of clinical response in several types of cancer. Despite the tremendous progress achieved in TIL and gene-modified T-cell therapy, tumor specimens may fail to give rise to viable TIL cultures and a high proportion of melanoma patients treated with either TIL or gene-modified T cells are nonresponders. A promising route for improving the clinical outcomes of TIL or gene-modified T-cell therapy is the enhancement of the functional properties of TIL or T-cell cultures via genetic modifications. However, until recently the traditional methods have yielded mediocre transduction efficiency.

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We have recently been able to vastly improve the efficiency of retroviral transduction of TILs [3], and thus, we present here protocols for their reproducible, time-efficient, and high-yield preparation. We also describe methods for transient transgene expression in TILs by mRNA transfection as well as viral transduction and mRNA transfection protocols for peripheral T cells and cell lines.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water) and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated). Diligently follow all waste disposal regulations. We do not add sodium azide to reagents.

Conduct all laboratory procedures under GMP conditions in a Class 10,000 clean laboratory using GMP-approved reagents.

2.1 Preparation of TIL Cultures

Basic complete medium (CM): Roswell Park Memorial Institute (RPMI) 1640 medium (e.g., Lonza, Verviers Sprl, Belgium) supplemented with 10% human AB serum (Valley Biomedical, Winchester, VA, or Gemini Bio, West Sacramento, CA) or heat-inactivated serum from 5 to 6 donors of any blood type obtained from a local blood bank.

2.1.1 Establishment of TIL Cultures

1. Enzyme-containing medium: RPMI 1640 medium containing 1 mg/mL collagenase (Sigma-Aldrich, Rehovot, Israel), 100 mg/mL hyaluronidase type V (Sigma-Aldrich), 30 U/mL DNase I type IV (Sigma-Aldrich), penicillin (100 U/mL)–streptomycin (100 μg/mL) (Pen/Strep; Biological Industries, Israel), 50 μg/mL gentamycin, and 1 mM L-glutamine (Lonza).
2. hrIL-2 (Peprotech; Rehovot, Israel) Cat. No. 200–02, dissolved in sterile water, 100 μg/mL stock, make aliquots, and store at −20 °C.
3. CM for establishment of TIL cultures (eCM): basic CM with the addition of Pen/Strep, 50 μg/mL gentamycin, 25 mM HEPES, pH 7.2, 0.25 μM 2-mercaptoethanol, and 3000 IU/ml human recombinant IL-2 (hrIL-2) (Chiron Novartis, New Jersey, USA).
4. Melanoma culture medium: eCM without IL-2.

2.1.2 Expansion of TIL Cultures

CM for initial rapid expansion phase (InRepCM): 50% eCM, supplemented with 50% AIM-V medium (Invitrogen, CA, USA), 30 ng/mL anti-CD3 monoclonal antibody (OKT3; Miltenyi Biotec, Bergisch Gladbach, Germany), and 3000 IU/mL hrIL-2- (Chiron Novartis, New Jersey, USA).

2.2 Preparation of PBMCs, T Cells, and T-Cell Lines

2.2.1 Activation of PBMCs

1. Cloudz™ Human T Cell Activation Kit (R&D Systems, Minneapolis, USA; Cat. No. CLD001). This kit includes microspheres composed of an alginate-based hydrogel functionalized with anti-CD3 and anti-CD28 antibodies. *See* **Note 1**.
2. T-cell culture medium: RPMI 1640 medium without L-glutamine, 10% fetal bovine serum (FBS), 2% Glutamax (an alternative to L-glutamine, with increased stability; Gibco [Thermo Fisher Scientific]), 10% HEPES, 0.1% β-mercaptoethanol, and 50 ng/mL hrIL-2. *See* **Notes 2** and **3**.
3. T-cell culture medium without hrIL-2.

2.2.2 Preparation of T Cells from PBMCs

1. Anti-human CD8 Magnetic Particles (BD Biosciences, Caesarea, Israel, Cat. No. 557766). *See* **Note 4** for alternatives.
2. BD IMag™ Buffer (10×) (Cat. No. 552362).
3. MagCellect Magnet (BD Biosciences, Cat. No. MAG997).

2.2.3 T-Cell Substitutes

For research purposes, cell lines such as T-cell hybridomas can be used as substitutes for freshly prepared T cells. Freshly isolated human T cells and TILs are prepared as described below. Examples of mouse T-cell hybridomas:

- **B3Z:** An H-$2^{K/b}$ $OVA_{257–264}$-H-$2K^{b}$-specific hybridoma, harboring the nuclear factor of activated T-cells (NFAT)–lacZ-inducible reporter gene for T-cell activation. The cells were a kind gift from Dr. N. Shastri, University of California, Berkeley.
- **BUSA14:** An H-$2^{K/b}$ $gp100_{25–33}$-H-$2D^{b}$-specific, NFAT–$lacZ^{+}$ hybridoma. The cells were a kind gift from prof. Lea Eisenbach, Weizmann Institute of Science, Israel.
- **CHIB2:** An H-$2^{K/NOD}$ $InsB_{15–23}$-H-$2K^{d}$-specific, NFAT–$lacZ^{+}$ hybridoma. The cells were a kind gift from Dr. Susan Wong, University of Bristol, UK.

Growth medium for melanoma and T-cell hybridoma cell lines: RPMI 1640 medium supplemented with 10% serum (either human for clinical use or FBS for experimental use only), Pen-Strep, 2 mM L-glutamine, 1 mM sodium pyruvate, and 25 mM HEPES.

2.3 Genetic Modification of TILs, PBMCs, T Cells, or Cell Lines

2.3.1 Viral Transduction

1. Dulbecco's Modified Eagle Medium (DMEM).
2. T-cell culture medium (see above).
3. 0.25% trypsin–EDTA (1×) (Gibco).
4. JetPrime buffer (Polyplus-transfection® SA, Strasbourg, France).
5. JetPrime reagent (Polyplus-transfection® SA). *See* **Note 5** for alternatives.
6. Sodium hypochlorite (bleach).

Virus Preparation

7. A retroviral system for transduction including three plasmids. There are numerous suppliers of such systems, and the researchers should use one that fits their specific needs.
   1. A transfer plasmid containing the gene of interest (GOI).
   2. A plasmid containing the viral Gag-Pol gene.
   3. A plasmid containing the Env gene encoding for the protein forming the viral envelope.
8. HEK293T cells.
9. HEK293T cell culture medium: DMEM, 10% FBS, 1% sodium pyruvate, 0.5% Pen–Strep.

Preparation of RetroNectin-Coated Dishes

RetroNectin (Takara Bio Inc., Otsu, Japan) is a 63-kD fragment of recombinant human fibronectin fragment (also referred to as rFN-CH-296) that enhances the efficiency of lentiviral- and retroviral-mediated gene transduction. It is provided as a sterile 1 mg/mL solution.

Binding of Retrovirus to RetroNectin-Coated Plates

2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).

Retrovirus Transduction of T Cells and TILs

STIM medium: AIM-V medium supplemented with Pen–Strep, 2 mM L-glutamine, 300 IU/mL hrIL-2, and 5% human serum.

Maintenance of Transduced TILs

1. Medium for transduced TIL maintenance – days 7–11 of rapid expansion protocol (TILmM1): AIM-V medium supplemented with Pen–Strep, 2 mM L-glutamine, 3000 IU/mL hrIL-2, and 5% human serum.
2. Medium for transduced TIL maintenance – days 11–14 of rapid expansion protocol (TILmM2): AIM-V medium supplemented with Pen–Strep, 2 mM L-glutamine, and 3000 IU/mL hrIL-2. This medium is identical to TILmM1 except that it lacks human serum.

2.3.2 mRNA Transfection of T Cells and TILs

1. T-cell culture medium (*see* Subheading 2.2.1).
2. T-cell culture medium with 2 ng/mL hrIL-2 (for PBMCs after mRNA transfection).
3. TIL culture medium with 250 ng/mL hrIL-2 (TIL growth medium before transfection).
4. TIL culture medium with 40 ng/mL hrIL-2 (for TIL after mRNA transfection only for 24 h).
5. Opti-MEM medium (Thermo Fisher). This product contains insulin, transferrin, hypoxanthine, thymidine, and trace elements. These additional components allow for a reduction in serum supplementation by at least 50%.

2.4 Flow Cytometry to Assess Transduction/ Transfection Efficiency of TILs

FACS buffer: 0.05% sodium azide, 0.5 μM EDTA, and 0.5 g/L BSA (preferably Quality Level 300, e.g., Sigma Cat. No. 4503-100G) in 500 mL PBS lacking $CaCl_2$ and $MgCl_2$.

3 Methods

3.1 Preparation of TILs

3.1.1 Establishment of Unselected/Young TIL Cultures

The initiation of TIL and tumor cell line (TCL) cultures starts with the surgical resection of metastatic lesions, which are used in three different methods in the following order: (A) cultivation of untreated tumor tissue fragments, (B) cultivation of TILs and tumor cells obtained by enzymatic digestion of tumor tissue fragments, and (C) tissue remnant culture (TRC), i.e., cultivation of cell remnants left behind on the cutting board after tumor tissue fragments were collected for methods (A) and (B) (Figs. 1 and 2).

Immediately after surgical resection of the metastatic lesion(s), place them in a sterile container containing sterile saline or CM. Tumor size should be at least 1 × 1 cm to be able to achieve beneficial cell yield. *See* **Note 6**.

Cultivation of Fragments

1. Place the tumor tissue on a cutting board and cut it with a scalpel into 6–12 fragments. Each fragment should be 1–2 mM^3 in size, sampled from different areas of the resected tissue, and kept bathed in PBS or CM while they are on the board.
2. Transfer each fragment to an individual well of a 24-well plate and add 2 mL CM (containing 3000 UI/mL IL-2) to each well.

Cultivation of TILs and Tumor Cells Obtained by Enzymatic Digestion of Tumor Tissue Fragments

1. Cut the tumor tissue remaining from the procedure described in Subheading "Cultivation of fragments" with a scalpel into 1–5 mM^3 fragments.
2. Transfer the fragments with a sterile tool to a sterile container with enzyme-containing medium (approximately 50 mL per 1 cm^3 of tumor tissue).
3. Place a sterile magnetic stirrer in the container and wrap the container with tin foil to limit evaporation and contamination.
4. Stir the tumor fragments for 2–4 h at 37 °C or overnight at room temperature until the cell dispersion is sufficient.
5. Filter the cell suspension through a sterile cell strainer into 50-mL conical tubes. Collect up to 25 mL per tube; *see* **Note 7**.
6. Complete the volume with PBS to 50 mL.

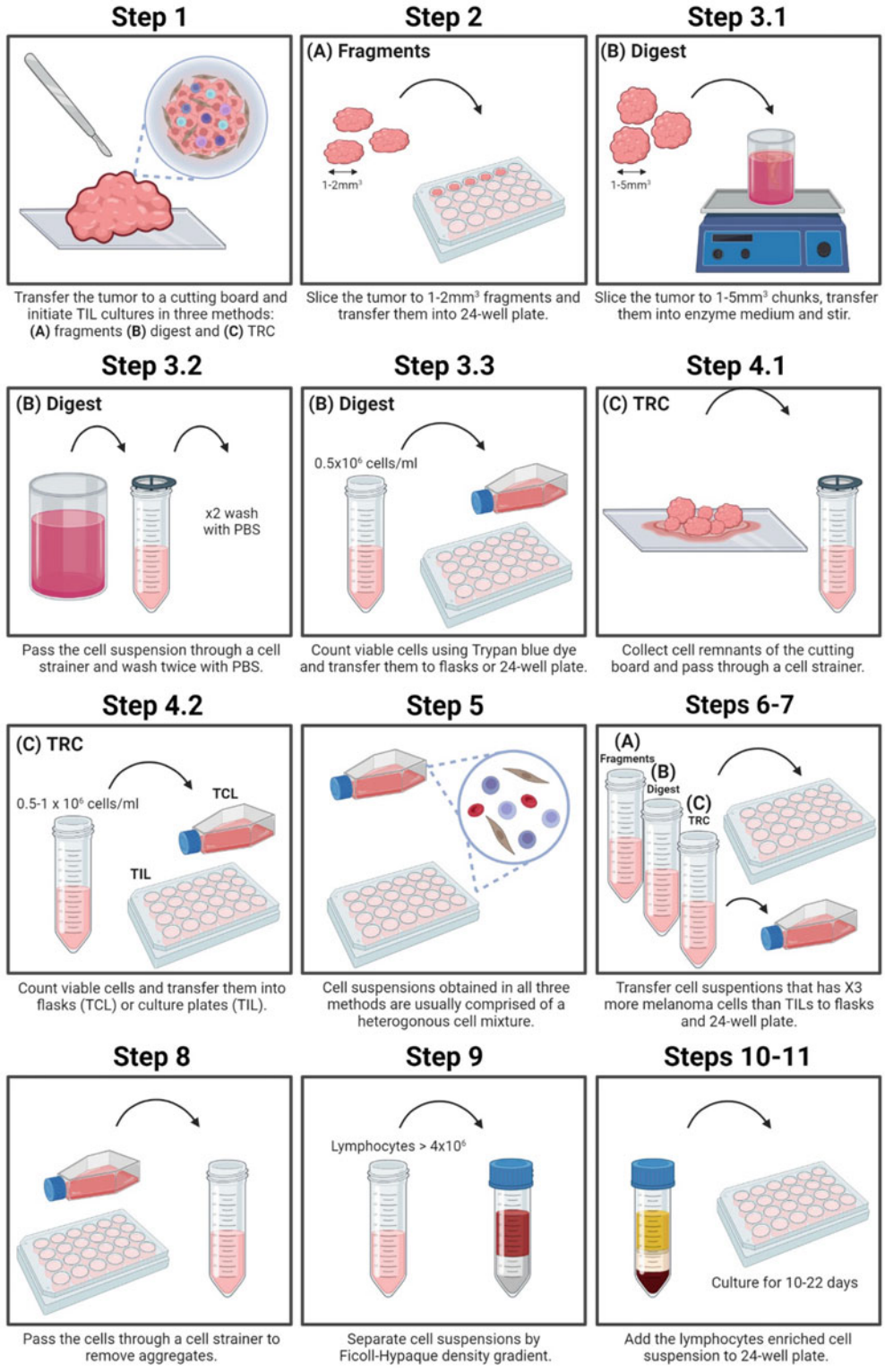
Step 1
Transfer the tumor to a cutting board and initiate TIL cultures in three methods: (A) fragments (B) digest and (C) TRC
Step 2
(A) Fragments
1-2mm³
Slice the tumor to 1-2mm³ fragments and transfer them into 24-well plate.
Step 3.1
(B) Digest
1-5mm³
Slice the tumor to 1-5mm³ chunks, transfer them into enzyme medium and stir.
Step 3.2
(B) Digest
x2 wash with PBS
Pass the cell suspension through a cell strainer and wash twice with PBS.
Step 3.3
(B) Digest
0.5x10⁶ cells/ml
Count viable cells using Trypan blue dye and transfer them to flasks or 24-well plate.
Step 4.1
(C) TRC
Collect cell remnants of the cutting board and pass through a cell strainer.
Step 4.2
(C) TRC
0.5-1 x 10⁶ cells/ml
TCL
TIL
Count viable cells and transfer them into flasks (TCL) or culture plates (TIL).
Step 5
Cell suspensions obtained in all three methods are usually comprised of a heterogonous cell mixture.
Steps 6-7
(A) Fragments
(B) Digest
(C) TRC
Transfer cell suspentions that has X3 more melanoma cells than TILs to flasks and 24-well plate.
Step 8
Pass the cells through a cell strainer to remove aggregates.
Step 9
Lymphocytes > 4x10⁶
Separate cell suspensions by Ficoll-Hypaque density gradient.
Steps 10-11
Culture for 10-22 days
Add the lymphocytes enriched cell suspension to 24-well plate.

Fig. 1 Establishment of TIL cultures. The initiation of TILs and tumor cell line (TCL) cultures starts with the surgical resection of metastatic lesions, which are used as the starting material in three different methods in

7. Centrifuge for 10 min at 1600 rpm (~500 × g) and remove the supernatant.
8. Resuspend the cell pellet in each tube in 50 mL PBS.
9. Centrifuge again for 10 min at 1600 rpm (~500 × g).
10. Resuspend the cell pellet in 10–50 mL PBS (depending on the size of the pellet) to count total viable cells.
11. Determine the total viable nucleated cell number by trypan blue dye exclusion.
12. Centrifuge for 10 min at 1600 rpm (~500 × g).
13. Aspirate the supernatant and resuspend the cell pellet in CM medium (including 3000 IU/mL IL-2) to obtain a concentration of 0.5×10^6 viable cells per ml.
14. Transfer the cells to a T75 (12–15 mL) or T175 (25–30 mL) culture flask or 24-well plate (2 mL per well).

Tissue Remnant Culture (TRC)

1. Place filter (cell strainer, 70 μm) over a 50-mL conical tube.
2. Collect the cell remnants left after methods Subheadings "Cultivation of fragments" and "Cultivation of TILs and tumor cells obtained by enzymatic digestion of tumor tissue fragments" from the cutting board and pass them through the cell strainer.
3. Centrifuge the tubes for 8 min at 300 × g.
4. Resuspend the cell pellet in 10–50 mL CM (including 3000 IU/mL IL-2) depending on the size of the pellet to count total viable cells.
5. Determine the total viable nucleated cell number by trypan blue dye exclusion.
6. Transfer the cells to a 24-well plate (for TIL growth) or flasks (for TCL growth), ($0.5–1 \times 10^6$ cells/mL).
7. After 1–3 days, transfer the cell suspension containing the TIL cells from the culture plate to a 24-well plate at 2 mL per well.
8. Change the medium in the flask to melanoma culture medium for melanoma growth.

Fig. 1 (continued) the following order: (**a**) cultivation of untreated tumor tissue fragments (**steps 1** and **2**), (**b**) cultivation of TILs and tumor cells obtained by enzymatic digestion of tumor tissue fragments (**steps 3.1–3.3**), and (**c**) tissue remnant culture (TRC), i.e., cultivation of cell remnants left behind on the cutting board after tumor tissue fragments are collected for methods (**a**) and (**b**) (**steps 4.1–4.2**). **Steps 5–11** depict the cultivation and isolation of lymphocytes and melanoma cells

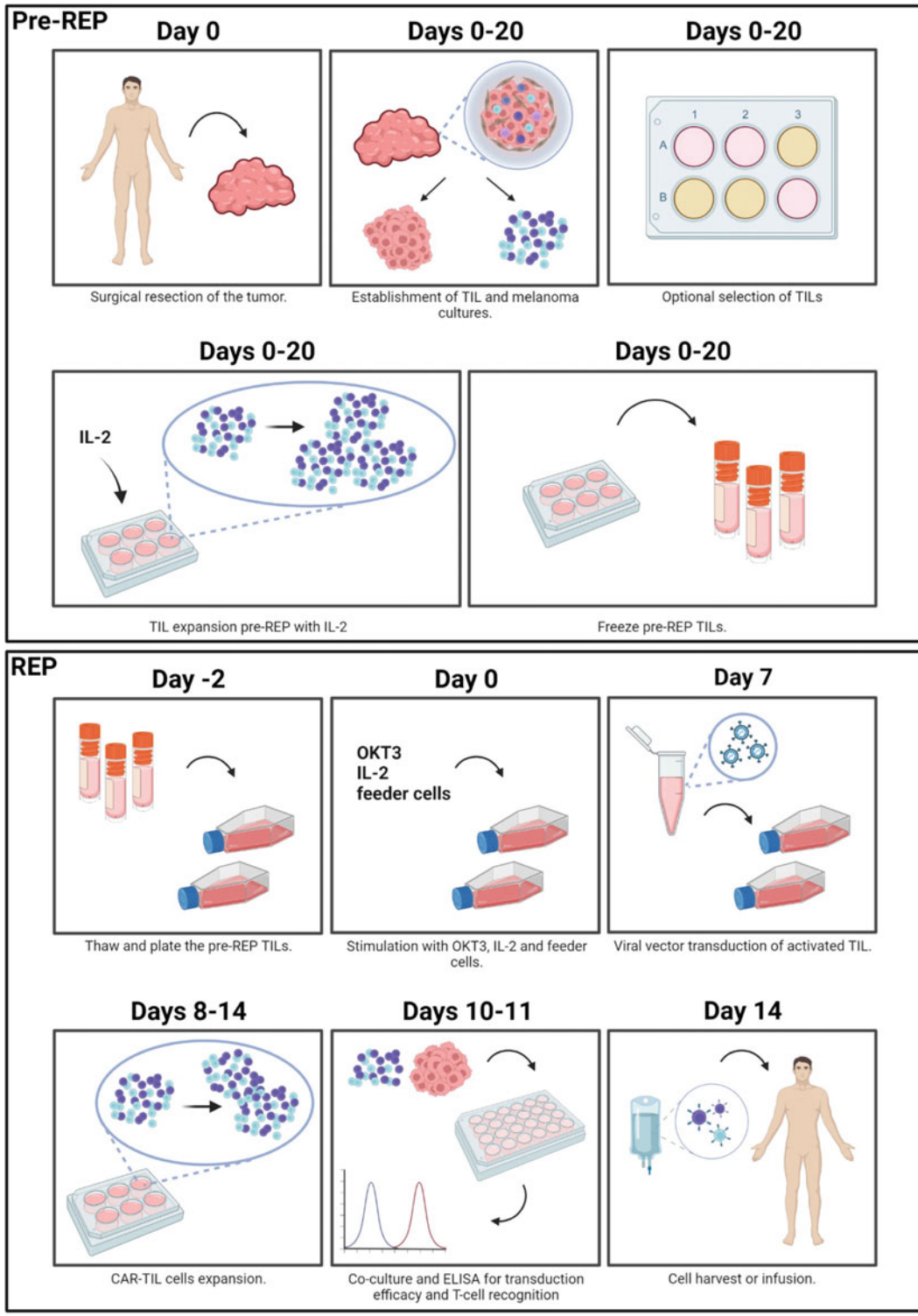

Fig. 2 TIL transduction. TIL transduction starts with surgical resection of metastatic lesions, the establishment of TILs and tumor cell line (TCL), such as melanoma, and TIL expansion employing activation with added interleukin 2 (IL-2). At this point, the activated cells may be stored or they can immediately be subject to the rapid expansion protocol (REP), including activating with anti-CD3 antibodies (OKT3), IL-2, and feeder cells; viral transduction with a GOI, such as CARs; and expansion of the transduced TILs. The melanoma cells may be used as test target cells for the transduced TILs

Pooling of Cells Obtained from the Three Methods Described Above and Separation of TILs and Melanoma Cells

The single-cell suspensions obtained by fragmentation, enzymatic digestion, or TRC usually comprise a heterogonous cell mixture of lymphocytes, melanoma cells, erythrocytes, fibroblasts, and cell debris. Lymphocytes and melanoma cells are separated according to the inherent tendency of lymphocytes to remain in suspension and melanoma cells' tendency to adhere to growth-vessel surfaces. Thus, a large amount of melanoma cells in the single-cell suspension requires a growth vessel with a large surface area, such as a tissue culture flask. Furthermore, the rate of expansion of TILs at this stage of the growth phase is higher in 24-well plates than in tissue culture flasks, and therefore, the single-cell suspension is divided into 24-well plates and tissue culture flasks as follows:

1. Transfer *about three-quarters of* the single-cell suspensions that contain at least three times more melanoma cells than TILs into tissue culture flasks (5×10^6 or less melanoma cells per T25 flask) and about a quarter of the cell suspensions into a 24-well plate (approximately 0.25×10^6 melanoma cells/mL/well). If the single-cell suspensions have substantially less than three times more melanoma cells than TILs, seed most of the single-cell suspension in a 24-well plate.

   After overnight incubation, the cell suspensions (from T25 flasks and 24-well plates) will contain nonadhered lymphocytes amongst other cells.

2. Pool the nonadhered lymphocytes and other cells *(from T25 flasks and 24-well plates)* and pass through a 70-mM cell strainer to remove large aggregates of erythrocytes and cell debris. Note that the melanoma cells adhere to the wells and can optionally be further cultivated for use as test target cells for TILs.

3. Count the number of nonadhered lymphocytes. If the total lymphocyte number exceeds approximately 4×10^6 cells, proceed to **step 4** or **5**. If the total lymphocyte number is less than 4×10^6 cells, plate the cells in a 24-well plate to obtain a single, dense layer of nonadhered cells. After 5–7 days, lymphocytes usually start proliferating. Once a cell density of approximately 4×10^6 lymphocytes is reached, go to **step 4** or **5**.

4. If the cell suspension contains at least ten times more erythrocytes than lymphocytes or has massive amounts of cell debris, fractionate the cell suspension by Ficoll-Hypaque density gradient, and add the obtained cell suspension, enriched for lymphocytes, to the wells containing adhered melanoma cells (*see* **step 2**) or to a new 24-well plate (1×10^6 lymphocytes/2 mL/well).

5. Cells obtained in **step 3** or **4** are cultured throughout the entire establishment process in CM (containing 3000 IU/mL hrIL-2). Once the lymphocytes start proliferating (approximately

1 week after surgery), they are maintained at a concentration of 0.5–2 × 10^6 per mL (2 mL per well). TIL cultures grow optimally in the 24-well plate. A young-TIL culture can be considered established when all melanoma cells are eliminated and a cell number of at least 5 × 10^7 TILs is reached. This process requires about 10–22 days. Short-term cultured TILs can then immediately be cryopreserved or used directly for further large-scale expansion.

3.1.2 Rapid Expansion Protocol (REP) of TILs

This section describes the standard rapid expansion protocol of TIL cells for clinical use at a smaller scale [1, 2] (Fig. 2).

1. Thaw TILs in eCM with 3000 IU/mL hrIL-2 and allow to rest for a period of 2 days at a concentration of 1.0 × 10^6/mL in a 24-well plate, or use freshly isolated TILs from **step 11** in Subheading 3.1.1 "Establishment of TIL Cultures."
2. Initiate a [mini-scale] REP by stimulating TILs by adding 30 ng/mL OKT3, 3000 IU/mL hrIL-2 (using InRepCM) and irradiated PBMC from non-related donors as feeder cells (5000 rad, TIL to feeder cells ratio = 1:100) in 50% CM and 50% AIM-V medium to a T25 flasks.
3. Harvest the TIL on the day of viral transduction (REP day 7), count, and adjust to a concentration of 0.5 × 10^6/mL in CM with 3000 IU/mL hrIL-2. Use the cells immediately or store them at 80 °C for 48 h, and then transfer to storage in liquid N_2.
4. Genetically modify the TILs as described below (Subheading "Retrovirus transduction of T cells and TILs" for viral transduction or Subheading 3.3.2 for mRNA transfection).

3.2 Preparation of PBMCs, T Cells, or T-Cell Lines

3.2.1 Activation of PBMCs

Cell Culture Setup and Activation

1. Thaw PBMCs into a 50-mL tube containing 20 mL T-cell culture medium without hrIL-2 or PBS containing 10% fetal calf serum (FCS) or FBS.
2. Count cells and centrifuge at 1250 rpm for 10 min at room temperature.
3. Resuspend cells in T-cell culture medium and transfer to 6-well plates.
4. Mix Cloudz CD3/CD28 microspheres by vortexing the vial for 5–10 s immediately before use. *See* **Note 1.**
5. Add 20 μL of Cloudz CD3/CD28 microspheres for every 1 × 10^6 PBMCs.
6. Gently mix cells with Cloudz CD3/CD28 microspheres by shaking or rocking for approximately 30 s.
7. Culture cells in PBMC growth medium in a humidified incubator (37 °C, 5% CO_2) for 2–3 days.

Cell Release by Dissolution of Cloudz CD3/CD28 Microspheres and Cell Collection

1. After 2–3 days, add 6× Release Buffer from the Cloudz kit directly into the cell culture medium at a 1:6 dilution (i.e., add 8 mL of 6× Release Buffer directly into a cell culture vessel containing 40 mL of culture medium). After the addition of the buffer, agitate the sample to ensure Cloudz CD3/CD28 microspheres are completely dissolved (approximately 5 min).
2. Collect T cells by centrifugation (1250 rpm for 10 min at room temperature).
3. Remove the supernatant. Resuspend the cell pellet in T-cell culture medium and seed in a 6-well plate.
4. Incubate the plate in a 37 °C, 5% CO_2 incubator for 24 h.

3.2.2 Preparation of T Cells from PBMCs (Magnetic Labeling; Fig. 3)

1. Dilute BD IMag™ Buffer 1:10 with sterile distilled water.
2. Count the activated PBMCs (**step 4** in Subheading "Cell release by dissolution of cloudz CD3/CD28 microspheres and cell collection"), wash them with 20 mL of 1× BD IMag™ buffer, and carefully aspirate all the supernatant.
3. Vortex the BD IMag™ anti-human CD8 magnetic particles and add 50 μL of particles for every 10^7 total cells.
4. *Mix thoroughly.* Incubate at room temperature for 30 min.
5. Bring the BD IMag™-particle volume up to 1–8 × 10^7 cells/mL with 1× BD IMag™ buffer and immediately place the tube on the cell separation magnet. Incubate for 8 min.
6. With the tube on the cell separation magnet, carefully aspirate off the supernatant. This supernatant contains the CD8-negative fraction, i.e., CD4 cells. The cells bound to the magnetic particles constitute the CD8-positive cell fraction.
7. Remove the tube from the cell separation magnet and add 1 mL of 1× BD IMag™ buffer to the remaining cells and bring the volume up to 1–8 × 10^7 cells/mL. Gently resuspend cells by pipetting up and down and return the tube to the cell separation magnet for another 4 min.
8. With the tube on the cell separation magnet, carefully aspirate off the supernatant to the same tube as in **step 6**.
9. Repeat **steps 7** and **8**.
10. After the final wash step, resuspend the CD8-positive cell fraction with T-cell culture medium (containing 50 ng/mL hrIL-2) and seed in a 6-well plate.
11. Centrifuge the CD8-negative fraction (i.e., CD4+ T-cell fraction) and resuspend with T-cell culture medium and seed in a 6-well plate.
12. Incubate the plate containing T cells for at least 24 h before the next procedure in 37 °C, 5% CO_2 incubator.

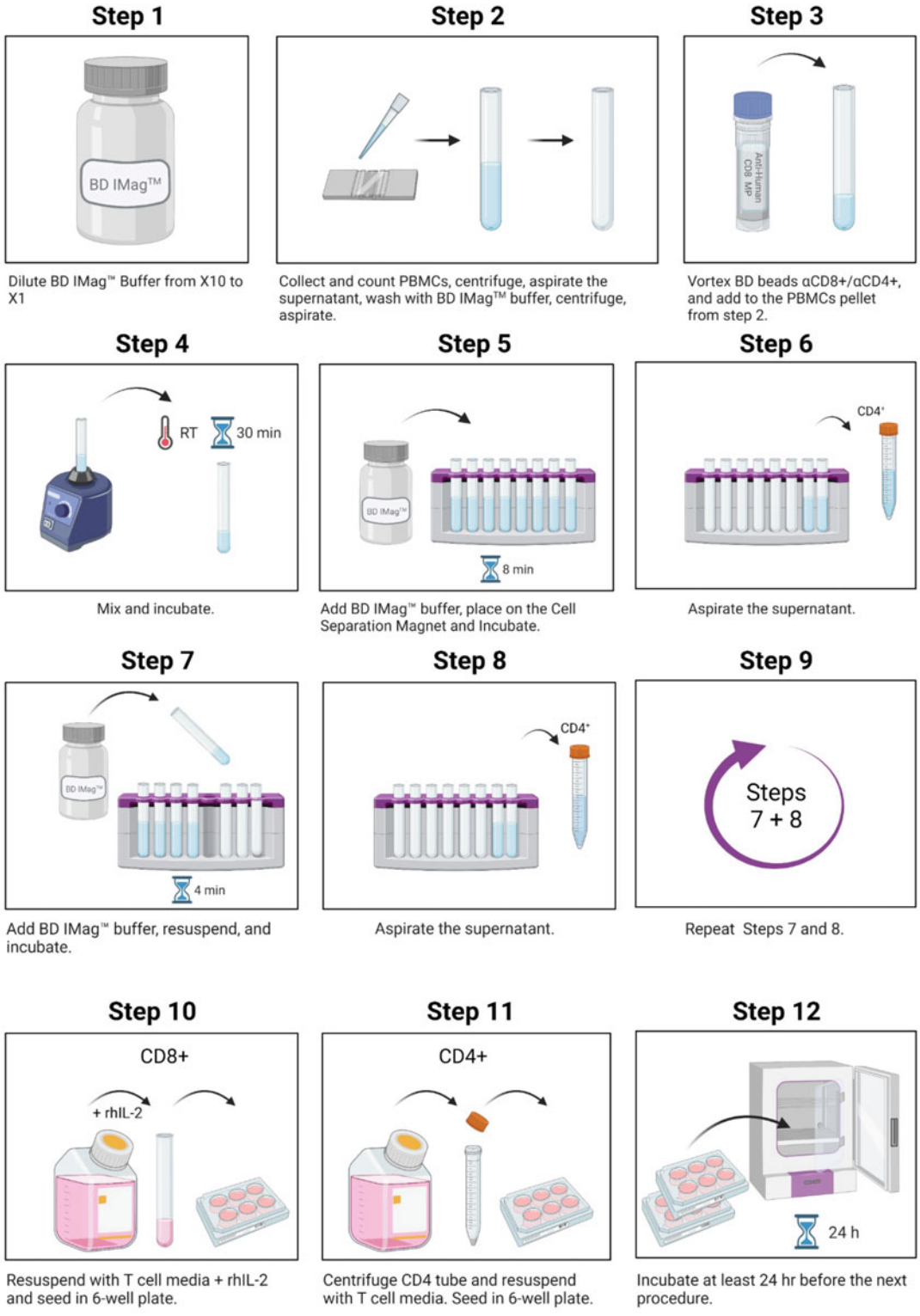
Step 1
BD IMag™
Dilute BD IMag™ Buffer from X10 to X1
Step 2
Collect and count PBMCs, centrifuge, aspirate the supernatant, wash with BD IMag™ buffer, centrifuge, aspirate.
Step 3
Anti-Human CD8 MP
Vortex BD beads αCD8+/αCD4+, and add to the PBMCs pellet from step 2.
Step 4
RT
30 min
Mix and incubate.
Step 5
BD IMag™
8 min
Add BD IMag™ buffer, place on the Cell Separation Magnet and Incubate.
Step 6
CD4+
Aspirate the supernatant.
Step 7
BD IMag™
4 min
Add BD IMag™ buffer, resuspend, and incubate.
Step 8
CD4+
Aspirate the supernatant.
Step 9
Steps 7 + 8
Repeat Steps 7 and 8.
Step 10
CD8+
+ rhIL-2
Resuspend with T cell media + rhIL-2 and seed in 6-well plate.
Step 11
CD4+
Centrifuge CD4 tube and resuspend with T cell media. Seed in 6-well plate.
Step 12
24 h
Incubate at least 24 hr before the next procedure.

Fig. 3 Separation of T cells from PBMCs. This procedure is based on the positive selection of $CD8^+$ T cells and the negative selection of $CD4^+$ T cells from an activated PBMC population. By mixing the cell suspension with anti-human CD8 magnetic particles, $CD8^+$ T cells bind to the magnetic particles and are separated from the unbound $CD4^+$ T cells with a magnet. The $CD4^+$ T cells are collected from the supernatant and the $CD8^+$ T cells are separated from the magnetic particles. "BD beads αCD8+/αCD4+," addition of magnetic beads with anti-CD8 or anti-CD4 antibodies; "rhIL2," recombinant human IL-2

3.2.3 Preparation of T-Cell Lines

1. Thaw a cell line, such as the one suggested in Subheading 2.2.3.
2. Culture the cells in growth medium for melanoma and T-cell hybridoma cell lines.

3.3 Genetic Modification of TILs, PBMCs, T Cells, or Cell Lines (Fig. 4)

3.3.1 Viral Transduction

Virus Preparation

Day -4 (4 days before transfection of the cells): Thawing of HEK293T cells.

1. Thaw HEK293T cells in 10 mL HEK293T cell culture medium.
2. Thaw PBMCs and activate them in a 6-well plate as described above in Subheading 3.2.1.
3. Incubate at 5% CO_2, 37 °C for 72 h.

Day -1: Seeding of HEK293T cells

1. Seed HEKT293T cells in 6-well plates in 2 mL HEKT293T cell culture medium. The aim is to bring the cells to about 60–70% confluence on day 0. In order to facilitate this, seed the cells at different cell densities in separate plates, e.g., 150,000 cells/well, 200,000 cells/well, and 250,000 cells/well. The plate in which the cells reach the appropriate cell density will be selected on day 0.
2. Incubate at 5% CO_2, 37 °C incubator for 24 h.
3. Seed the activated PBMCs from **step 3** above (day-4) in a 6-well plate, and incubate for 48 h at 5% CO_2, 37 °C.

Day 0: Transfection of HEK293T cells with a retroviral transduction system

1. For each transfection, add to an Eppendorf tube in this order:
   1.1 200 μL JetPrime buffer (for 1 well in a 6-well plate).
   1.2 Plasmids of a retroviral system for transduction including at least (1) a transfer plasmid containing the GOI, (2) a plasmid containing the viral Gag-Pol gene, and (3) a plasmid containing the Env gene.
2. Vortex 10 s and spin-down.
3. Vortex jetPRIME® reagent for 5 s and spin down before use.
4. Add 4 μL jetPRIME® reagent.
5. Vortex 10 s and spin-down briefly.
6. Incubate for 10 min at room temperature.
7. Add 200 μL of transfection mix per well dropwise onto the HEK293T cells (60–70% confluent) and distribute evenly.
8. Gently rock the plates back and forth and from side to side.
9. Incubate the plate at 5% CO_2, 37 °C for 4 h.

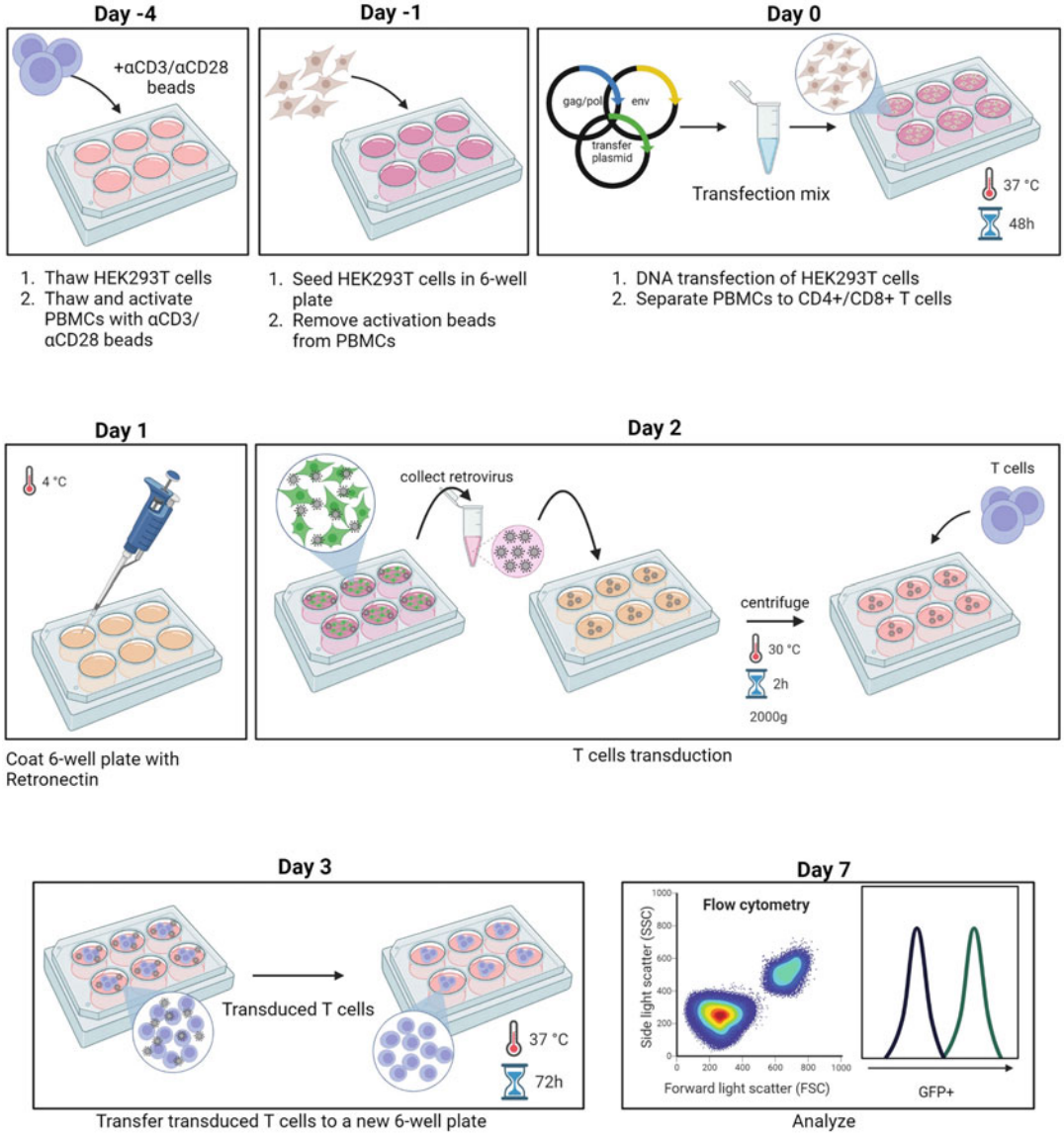

Fig. 4 Overview of the transduction process. Target cells intended for genetic manipulation, either TILs or PBMCs, are activated in 6-well plates. HEK293T cells are cultivated in parallel and transfected with plasmids of a retroviral system for transduction including at least (1) a transfer plasmid containing the GOI, (2) a plasmid containing the viral Gag-Pol gene, and (3) a plasmid containing the Env gene. On day 1, a 6-well plate is coated with RetroNectin. On day 2, the supernatant, which contains retroviral particles, is collected from the HEK293T cell plate and added to the RetroNectin-coated plate. The target cells are then added to the RetroNectin-bound viral particles and are transferred to a new six-well plate on Day 3. "+αCD3/αCD28" – addition of beads with anti-CD3 and anti-CD28 antibodies

10. Replace the medium with fresh medium.
11. Incubate at 5% CO_2, 37 °C incubator for 48 h.
12. Optionally, harvest the supernatant containing the retrovirus and store it at −80 °C.

Preparation of RetroNectin-Coated Dishes

Day 1

Non-treated, cell culture-grade tissue culture plates or dishes should be used in this step.

1. Before coating, prepare a RetroNectin solution (20–100 μg/mL) by diluting with sterile PBS.
2. Add 2.25 mL/well of RetroNectin solution at a concentration of 20 μg/mL in a 6-well plate (since each well is 35 mM in diameter [≅9 cm^2], this gives a density of 5 ≅μg/cm^2). *See* **Notes 8** and **9**. Dispense an appropriate volume of sterile RetroNectin solution into each plate.
3. Allow the plate to stand for 2 h at room temperature or at 4 °C overnight. *See* **Note 10**.

Binding of Retrovirus to RetroNectin-Coated Plates

Day 2: Transduction

1. Preheat centrifuge by running it empty at 2000 *g* at 32°C.
2. Prepare a blocking solution of sterile 2% BSA (Fraction V) in PBS. For one 6-well plate – 0.3 g BSA in 15 mL PBS.
3. Aspirate the RetroNectin solution from the 6-well plate. Make sure that the wells are not left without liquid by aspirating no more than 3 wells before adding the blocking solution (*see* **step 4**). *See* **Note 11.**
4. Block with an appropriate volume of blocking solution: 2 mL for each well of a 6-well plate of sterile 2% BSA in PBS.
5. Allow the plate to stand at room temperature for 30 min.
6. While waiting for **step 5**, harvest the retroviral supernatant from HEK293T cells from the 6-well plate and transfer it to a 15-mL tube (**step 11** on day 0) or thaw the stored retroviral supernatant.
7. Centrifuge the 15-mL tube with the viral supernatant from **step 6** at 10 min at 1000 rpm.
8. While centrifugation is ongoing in **step 7**, remove the BSA solution (blocking solution) from the RetroNectin 6-well plate and wash the plate once with an appropriate volume (2 mL/well) of PBS, leaving the wash solution on the plate.
9. Aspirate the wash solution just before the viral supernatant is added.

10. Add 2–4 mL retrovirus solution from **step 7** to the RetroNectin-coated plate. If the target cells that will be added to the retrovirus-coated wells at the next step (Subheading "Retrovirus transduction of T cells and TILs") are TILs, optionally dilute the retrovirus solution 1:2 in STIM medium before transfer to the RetroNectin-coated plate. *See* **Note 12.**
11. Cover with plastic wrap.
12. Place the plate in the pre-warmed centrifuge and centrifuge for 2 h at 32 °C at 1000–2000 *g* to facilitate binding of virus particles with RetroNectin reagent. In the specific case of 6-well plates, the optimum conditions are to centrifuge at 2000 *g* for 2 h at 32 °C.
13. Prepare the target TIL or T cells while the retrovirus particles are binding to the RetroNectin reagent-coated plates: *See* **Note 13** and below at Subheading "Retrovirus transduction of T cells and TILs" for details.
14. Collect the target cells from their storage plates and count the number of living cells.
15. Suspend the target cells in the growth medium: Suspend T cells in T-cell culture medium at a density of 0.5×10^6 cells/mL, or suspend TILs in STIM medium at a density of 2×10^6 TILs.
16. Remove the plate with the RetroNectin-bound virus particles from the centrifuge.
17. Aspirate the viral supernatant from the plates and transfer into a 50-mL tube containing a small amount of sodium hypochlorite (to inactivate the remaining retroviral particles), but leave a residual volume in the wells (to avoid both contact and drying of the wells). Immediately proceed to **step 1** of Subheading "Retrovirus transduction of T cells and TILs" for retrovirus transduction or *see* **Note 14**.

Retrovirus Transduction of T Cells and TILs

1. Immediately add target cells to the RetroNectin-coated plates:
   (a) Add T cells at a density of 0.5×10^6 cells/mL (3×10^6 cells in 6 mL of T-cell culture medium). The T cells are used directly after **step 4** of Subheading "Cell release by dissolution of cloudz CD3/CD28 microspheres and cell collection" or after **step 12** of Subheading 3.2.2: "Preparation of T cells from PBMCs."
   (b) Add TILs at a density of 2×10^6 TILs in a medium for TIL retrovirus transduction (STIM medium) containing 300 IU/mL rhIL-2 to each RetroNectin-coated well. The TILs are used either directly after harvest

(Subheading 3.1.2: "Rapid Expansion Protocol (REP) of TILs") or, if frozen, are thawed 2 days before use.

2. To promote contact between the target cells and viral particles, plates can be centrifuged after adding the cells: Wrap plate, and then centrifuge for 10 min at 1500 rpm with the acceleration and brake set at 1. Make sure it is appropriately balanced!
3. Gently remove the plate from the centrifuge.
4. Check the cell density in a microscope. This is just a quality check to verify that the cells are seeded.
5. Incubate the plate in a 37 °C, 5% CO_2 incubator overnight (TILs or T cells) or for 2–3 days.

Maintenance of Transduced T Cells

Day 3

1. Transfer the transduced T-cell supernatant into a 15-mL tube.
2. Recover remaining nonadherent cells by washing the plate with PBS, collecting, and adding to the 15-mL tube.
3. Centrifuge the 15-mL tube at 1250 rpm for 10 min to recover the cells.
4. Resuspend the cells with 1 mL fresh T-cell culture medium and seed in a 6-well plate (total 4 mL medium per well).
5. Incubate at 37 °C for 72 h.

Day 6 and onward: Analyze the transduced T cells for transduction efficiency or activity (FACS, ELISA, killing assay).

Maintenance of Transduced TILs

1. Transfer transduced TILs to 6-well culture plates and maintain at a concentration of 0.5–2.0 × 10^6 cells/mL in TILmM1 medium with 300 IU/mL hrIL-2 and further expand until day 10–11.
2. On day 10–11, replace the TILmM1 medium with TILmM2 medium.
3. Day 10 and onward: Analyze the transduced TILs for transduction efficiency or activity (FACS, ELISA, killing assay).

3.3.2 mRNA Transfection of TILs and T Cells

The mRNA transfection of T cells is performed either directly after harvest (3 days post-separation of T cells after **step 12** of Subheading 3.2.2: "Preparation of T cells from PBMCs") or 1 week after thawing the PBMCs.

The mRNA transfection of TILs is performed either directly after harvest (Subheading 3.1.2: "Rapid Expansion Protocol (REP) of TILs") or, if frozen, 2 days (day 11–12 REP) after thawing them in TILmM2 with 3000 IU/mL hrIL-2, seeding in 6-well plate and incubating at 37 °C, 5% CO_2 for 2–3 days.

1. Pre-chill cuvettes on ice.
2. Count the TILs or T cells.
3. Collect the cells and wash them with cold Opti-MEM medium by discarding the supernatant and resuspending in fresh Opti-MEM medium, centrifugation, and repeat.
4. Resuspend in cold Opti-MEM medium at a final concentration of 1.6×10^7 cells/mL and keep on ice.
5. Mix 180 μL of cells with 10 μg mRNA in cold 2-mM cuvettes and using an electroporation device such as Gene Pulser Xcell (BioRad) or AgilePulse MAX System (BTX), electroporate under the following conditions: 1-ms square-wave pulse at 360–380 V.
6. Immediately transfer the cells to a 12-well plate that contains a 3 mL T-cell culture medium with 2 ng/mL hrIL-2.
7. Incubate the plate in a 37 °C, 5% CO_2 incubator for 24 h for downstream analysis or applications.

3.4 Flow Cytometry to Assess Transduction/ Transfection Efficiency of TILs

Transduction efficiency is determined 4 days after transduction (day 11 of REP) and 7 days after transduction (day 14 of REP) using flow cytometry by staining transduced cells with labeled antibodies targeting the GOI product expressed by successfully transduced cells. For example, CAR-expressing TILs are labeled with biotin-labeled, goat anti-mouse IgG, F (ab′)2-specific antibody (Jackson ImmunoResearch, West Grove, PA), and streptavidin (APC conjugated; BD Bioscience, San Jose, CA).

1. For analysis of TILs, wash the cells and resuspend them in cell staining buffer (BioLegend, San Diego, CA).
2. Incubate cells for 30 min with the antibodies on ice.
3. Wash in FACS buffer.
4. Measure using a FACS instrument, MACSQuant (Miltenyi Biotec). Samples may be analyzed using FlowJo software (FlowJo LLC, Ashland, OR). Cells are gated according to FCS/SSC and singlets.

4 Notes

1. Relevant alternatives are Dynabeads Human T-Expander CD3/CD28 (Thermo Fisher Scientific, 11141D) and DynaMag for Eppendorf or conical tubes (Invitrogen 12321D, or MACSiMAG separator, Miltenyi Biotec 130–092-168).

   Another option for activation: Place PBMCs at a concentration of 1.5×10^6 cells/ml in growth medium containing 250 ng/mL OKT3, 125 ng/mL anti-CD28 mAb, and

1000 IU/mL hrIL-2 and incubate for 72 h at 37 °C and 5% CO_2.

2. Medium should be filtered with a 0.2-micron filter unit.
3. Once hrIL-2 is added to the medium, the medium should be used within a week. It is recommended to make the T-cell medium without IL-2 and then take a portion of it to make hrIL-2 containing T-cell culture medium.
4. Alternative kits for T-cell enrichment:
   (a) EasySep Human CD4/CD8 T Cell Enrichment Kit (StemCell Tech 17,952/17968).
   (b) EasySep buffer (StemCell Tech 20,144).
   (c) EasySep Magnet for 15-ml round-bottom tubes (StemCell Tech 18,001).
   (d) MAGH102, MagCellect Human CD4+ T Cell Isolation Kit R&D (R&D Systems).
5. Alternative kits for virus preparation:
   (a) Xfect™ (Takara Bio).
   (b) FuGENE® HD Transfection Reagent (Active Motif).
6. It is important to receive the specimen in the laboratory within a few hours after surgery, bathed in sterile saline in a sterile container. Keep the tissue at room temperature if TIL preparation is initiated immediately; keep the tissue at 4 °C if it is delayed.
7. Alternatively, to accommodate a large volume of the cell suspension, one may filter it through a sterile metal mesh filter into one or larger 225-mL tubes.
8. If other plates are used, coat a plate using 20–100 μg/mL RetroNectin with a volume corresponding to a 4–20 μg/cm^2 plate area. (For 6-well plates the optimum density is 5 μg/cm^2.)
9. To avoid loss of RetroNectin fragment, do not filter-sterilize RetroNectin solution diluted with PBS.
10. The RetroNectin-coated plate can be sealed with parafilm and stored at 4 °C for up to 1 week.
11. You may discard the RetroNectin solution aspirated from the 6-well plate or store it for a second use.
12. In this method, some infection-inhibiting molecules may not be removed by washing with PBS. For this reason, the efficiency of gene transduction might be reduced. In such a case, it is recommended that the virus stock solution be used after dilution with growth medium. Optimization is required to determine the suitable dilution rate.
13. The target cells must be at the logarithmic growth phase and express integrin receptors VLA-4 and/or VLA-5.

14. Optionally wash the plate with an appropriate volume of PBS or PBS containing 0.1–2% albumin (BSA or HSA) before proceeding to **step 1** of Subheading "Retrovirus transduction of T cells and TILs" for retrovirus transduction.

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All figures were created with Biorender.com.

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Chapter 14

Transposon-Based Manufacturing of Human CAR-T Cells

Megan Tennant and Richard O'Neil

Abstract

In this chapter, the methodologies are outlined for generating CAR-T from PBMCs using transposon engineering. Additionally, some methods and guidance related to basic functional and phenotypic analysis are described. This methodology can be applied to manufacture and assess chimeric antigen receptors for preclinical applications targeting a variety of molecules.

Key words Adoptive cell therapy, CAR-T therapy, Transposon, PiggyBac, Transfection, T cell, Transposase, Immunotherapy, Lymphoma

1 Introduction

Adoptive cell transfer (ACT) is a revolutionary form of immunotherapy involving the infusion of lymphocytes for antiviral, antitumor, or anti-inflammatory purposes [1, 2]. By combining ACT with ex vivo cell culture and cellular engineering approaches, the field has rapidly advanced from a promise to practice leading to commercial approvals of chimeric antigen receptor (CAR) T cells to treat hematological cancers [3–5]. These clinical outcomes have progressed the field of CAR therapy and generated unprecedented interest in cell-based therapies, sparking large-scale preclinical studies and engagement of both biotechnology and pharmaceutical sector [6].

CARs are synthetically engineered receptors which function to redirect lymphocytes to recognize cells expressing a specific target antigen [7]. Most commonly, CARs are associated with cytotoxic T lymphocytes (CAR-T cells), resulting in vigorous T cell activation and powerful antitumor responses [8]. CAR-T therapy has been revolutionary as it has produced effective and durable clinical responses. Most notably, the use of anti-CD19 CAR-T cell therapy in B cell malignancies has shown unprecedented clinical success,

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resulting in the 2017 approval by the US Food and Drug Administration (FDA) [3–5].

Several modalities are established for engineering T cells into CAR-T cells. While T cells modified with gamma-retroviral or lentiviral vectors have shown remarkable efficacy in clinical trials, [9] there are several drawbacks to using viral approaches. Regarding clinical manufacture, the need for specialized manufacturing processes makes generation of clinical grade lentivirus very expensive and time consuming. In the preclinical setting, the complexity of lentiviral manufacturing reduces flexibility needed to test a variety of constructs and the high cost associated with viral vectors is inhibitory for rapid preclinical development. Several studies have recently explored the use of nonviral transfection-based approaches including the use of transposons as an alternative for CAR-T cell generation [10, 11].

Nonviral, plasmid-based transposon systems have emerged as an efficient method for stable genetic modification of human cells for therapeutic applications [10, 11]. A major benefit of transposon-based engineering is its flexibility and ability to move large transgenes [12] and to simultaneous deliver multiple transgenes [13]. Furthermore, plasmids encoding transposons can be easily manufactured in large quantities in most basic molecular biology laboratories affording more widespread experimentation. Another advantage of transposon systems is that they can be used for point-of-care manufacture of CAR-T cells drastically reducing manufacturing time, especially important in the context of acutely ill patients that may not survive the 4–6 week manufacture time required to make conventional CAR-T cells by lentiviral approaches [14]. Consequently, s*leepingBeauty* and *PiggyBac* transposon systems have been approved for use in human CAR-T cell clinical trials for a number of indications [15, 16].

In this chapter, the procedures are elaborated for generating human CD19 CAR-T cells using a transposon-based system. Furthermore, we describe strategies that can be deployed to conduct basic phenotypic and functional characterization of the manufactured cells. It is likely that these methods can be easily adapted for other CAR-T cell targets by leveraging different target-recognizing domains.

2 Materials

2.1 Plasmids (Fig. 1)

1. pRP[Exp]-mCherry-CAG > hyPBase (Vectorbuilder cat# VB900088-2876mrd) *PiggyBac* transposase plasmid.
2. pTpB-hCD19-41BB vector. Anti-CD19 chimeric antigen receptor (CAR) with 4-1BB costimulatory domain (*see* **Note 1**).

3. pTpB-hCD19-CD28 vector of anti-CD19 chimeric antigen receptor (CAR) with CD28 costimulatory domain (*see* **Note 2**).
4. pTpB-CMV-hCD19 mammalian expression vector for membrane bound human CD19 [17] (*see* **Note 3**).
5. ZymoPURE II Plasmid Maxiprep Kit (D4202).

2.2 PBMC Isolation from Human Peripheral Blood Sample

1. Fresh human PMBC.
2. Hanks' Balanced Salt Solution (HBSS) (1×).
3. Lympholyte® Mammal Cell Separation Media (Cedarline).
4. T cell media: advanced RPMI supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, and 1% Pen-Strep.
5. Recombinant IL-7.
6. Benchtop centrifugation instrument: Eppendorf 5810R; rotor: A-4-81 with 15/50-mL buckets.
7. 24-well cell culture plates (sterile, flat bottom).

2.3 Transfection

1. Amaxa Nucleofector (Lonza).
2. Human T cell Nucleofector Kit (Lonza).
3. T cell media (described previously).
4. 12-well cell culture plates (sterile, flat bottom).

2.4 Cell Culture and CAR-T Cell Expansion

1. T cell media (described previously).
2. $CD19^{+}$ K562 cell line.
3. Recombinant IL-2.
4. 24-well cell culture plates (sterile, flat bottom).

2.5 Cryopreservation of CAR-T Cells Post Expansion

1. Cryopreservation media: advanced RPMI supplemented with 40% human serum and 10% DMSO.
2. Microcentrifuge tubes with screw caps for cryopreservation.

2.6 Generation of HEK293Tscan-CD19+ Cells for Granzyme

1. Neon Transfection System (Thermo Fisher, USA).
2. pTpB-CMV-Tscan-hmbCD19 (Fig. 1).
3. $HEK293^{HLAnull}$ cells [18] (*see* **Note 15**).
4. 6-well cell culture plates (sterile, flat bottom).

2.7 HLA Null Granzyme Reporter Cells (HEK293Tscan-CD19+) Cell Culture

1. HEK-293Tscan-CD19+ cells.
2. HEK-293 T media: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%.
3. FBS, 2 mM Glutamax and 0.2% Pen/Strep.
4. 0.25% trypsin–EDTA.

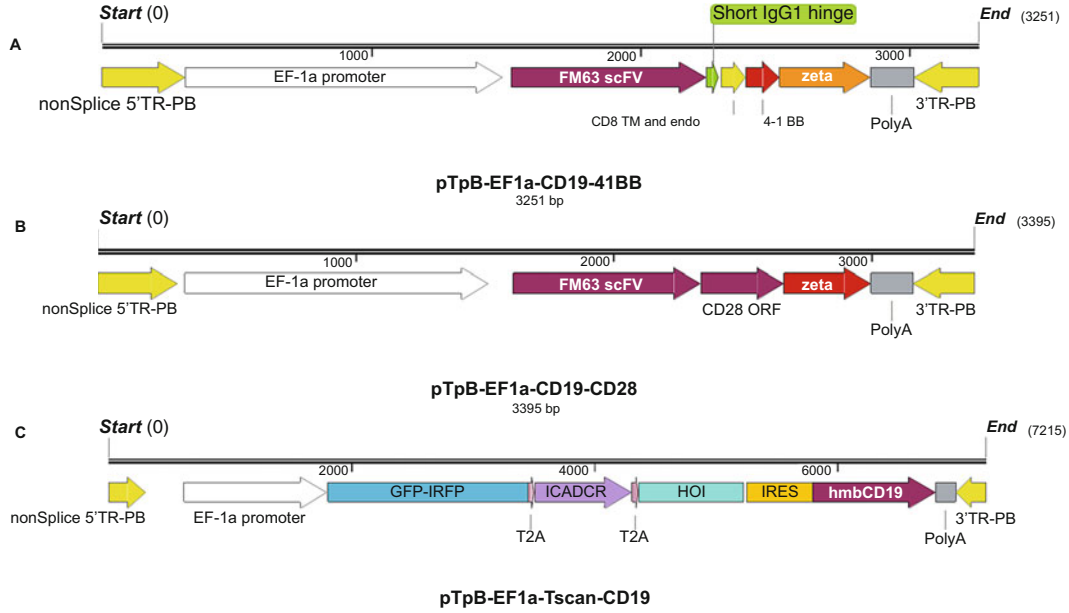

Fig. 1 Vector schematics. The *PiggyBac* transposase was used with the pTpB-hCD19-41BB, pTpB-hCD19-CD28, and the pTpB-Tscan-CD19 transposons. CMV, cytomegalovirus immediate early enhancer/promoter; *PiggyBac*, transposase; FM63 scFv, human CD19 target-recognizing domain; 4-1BB, 4-1BB intracellular signaling domain; CD28 ORF, CD28 transmembrane domain and signaling domain; GFP, green fluorescent protein reporter gene; ICADCR, caspase-resistant version of inhibitor of caspase-activated DNase (ICAD) transmembrane domain; HO1, heme oxegenase-1; IRES, internal ribosomal entry site; hmbCD19, truncated human membrane-bound CD19; WPRE, woodchuck hepatitis posttranscriptional regulatory element; PolyA, simian virus 40 late polyadenylation signal. Solid yellow arrows indicate *PiggyBac* inverted terminal repeat (TR) sequences

2.8 Flow Cytometry Analysis

1. Dulbecco's phosphate-buffered saline (DPBS) (1×).
2. BV421 mouse anti-human CD3 (BD#563797).
3. PE-Cy7 mouse anti-human CD4 (BD#560909).
4. APC mouse anti-human CD8 (BD#561952).
5. UltraComp eBeads compensation beads (Thermo Fisher, USA).
6. Staining buffer: 1× DPBS with 2.5% FBS.
7. Round-bottom polystyrene tubes (5 mL).
8. CytoFLEX LX flow cytometer.

3 Methods

3.1 Plasmid Construction and Preparation

1. Transfection-grade plasmids were prepared for the aforementioned constructs according to the manufacturer's protocol using endotoxin-free buffers and ZymoPURE II Plasmid Maxiprep Kits (ZYMO Research, USA) (*see* **Notes 4 and 5**).

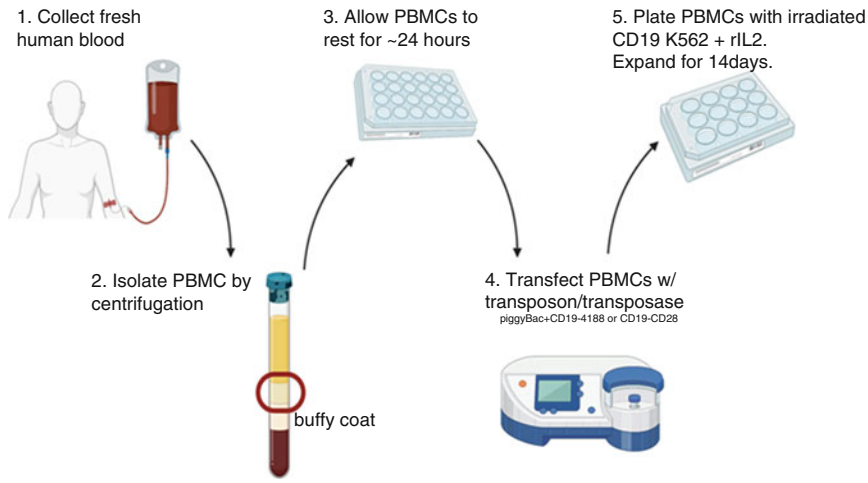
1. Collect fresh human blood
2. Isolate PBMC by centrifugation
buffy coat
3. Allow PBMCs to rest for ~24 hours
4. Transfect PBMCs w/ transposon/transposase
piggyBac+CD19-4188 or CD19-CD28
5. Plate PBMCs with irradiated CD19 K562 + rIL2. Expand for 14days.

Fig. 2 Schematic depicting the process of transposon-based manufacturing of human CAR-T cells

3.2 PBMC Isolation

1. On the day prior to transfection (day −1), obtain fresh human heparinized blood (*see* **Note 6**).
2. Dilute blood 1:1 with HBSS and layer mixture on top of the lymphocyte separation medium **(***see* **Note 7)** in 50-mL conical tubes using equal parts of the blood/HBSS mixture and lymphocyte separation medium.
3. Centrifuge at 800 × g for 20 min at 22 °C (*see* **Note 8**).
4. Collect the buffy coat later containing lymphocytes.
5. Wash twice with 10 mL of PBS at 500 × g for 3 min at 4 °C.
6. Resuspend cells in T cell media.
7. Count cells and determine viability before diluting to 2×10^6 viable cells/mL with T cell media + 10 ng/mL recombinant human IL-7 (4.4×10^5 IU/ug).
8. Plate 2 mL/well of cells in a 24-well plate and incubate overnight at 37 °C with 5% CO_2.

3.3 Stable Transfection of PBMCs

Perform transfection by following the guidelines of Amaxa® Human T Cell Nucleofector® Kit (Fig. 2). Below is a brief outline of the method for transfection.

1. The following morning (day 0), determine cell density and viability (*see* **Note 9**). Resuspend cells in a final solution containing 1×10^7 viable cells +5 ug of *PiggyBac* transposase (pRP [Exp]-mCherry-CAG > hyPBase) DNA + 5 ug of CD19-41BB or 5 ug of *PiggyBac* transposase DNA + 5 ug pTpB-CD19-CD28 in a total volume of 100 μL of Human T Cell Nucleofector® Solution.

2. Transfer the cell/DNA mixture into a certified cuvette ensuring that the sample covers the bottom of the cuvettes and there are no air bubbles (*see* **Note 10**). Close the cuvette.
3. Insert the cuvette containing the cell/DNA mixture into the Nucleofector® Cuvette Holder. Select the Nucleofector® Program U-014 and apply the program.
4. Once the program is completed, transfer the sample into a 12-well plate containing 2 mL of T cell media per well. Incubate transfected cells overnight at 37 °C with 5% CO_2.

3.4 Expansion of CAR-T Cells

1. On the first day after transfection (day 1), assess cell density and viability. Resuspend cells in T cell media at a final concentration of 2×10^6 cells/mL + 100 IU/mL rIL-2 (2×).
2. Plate transfected cells in a 24-well plate with 1 mL/well.
3. After plating transfected cells, count and resuspend previously irradiated (100gy) $CD19^+$ K562 cells at a final concentration of 5×10^4 in T cell media (*see* **Note 11**).
4. Add 1 mL of resuspended K562 cells on top of transfected cells in the 24-well plate for a final volume of 2 mL/well +50 IU/mL rIL-2 and a final responder to stimulator ratio of 40:1.
5. Expand cells at 37 °C with 5% CO_2 for 14 days while monitoring the growth of the transfected cells (Fig. 3), maintaining cell concentration under 2×10^6 cells/mL.
6. As needed, split and feed cells with fresh T cell media + 50 IU/mL rIL-2.

3.5 Cryopreservation of Expanded CAR-T Cells

After 14 days of expansion, CAR-T cells were cryopreserved for future analysis.

1. Harvest cells and wash twice with PBS at 500 × g for 3 min at 4 °C.
2. Access cell density and viability and aliquot into cryopreservation media at 1×10^6 CAR-T cells per tube.
3. Place tubes in −80 °C overnight before placing into liquid nitrogen for long-term storage (*see* **Note 12**).

3.6 CAR-T Cell Phenotype Analysis

Previously expanded CAR-T cells are analyzed for phenotype.

1. Locate an aliquot of previously cryopreserved CD19–41BB and CD19–CD28 CAR-T cells.
2. Thaw cells quickly in a 37 °C water bath.
3. Mix and add entire contents of the vial to 9 mL of T cell media and mix gently.
4. Centrifuge at 500 × g for 3 min at 4 °C.

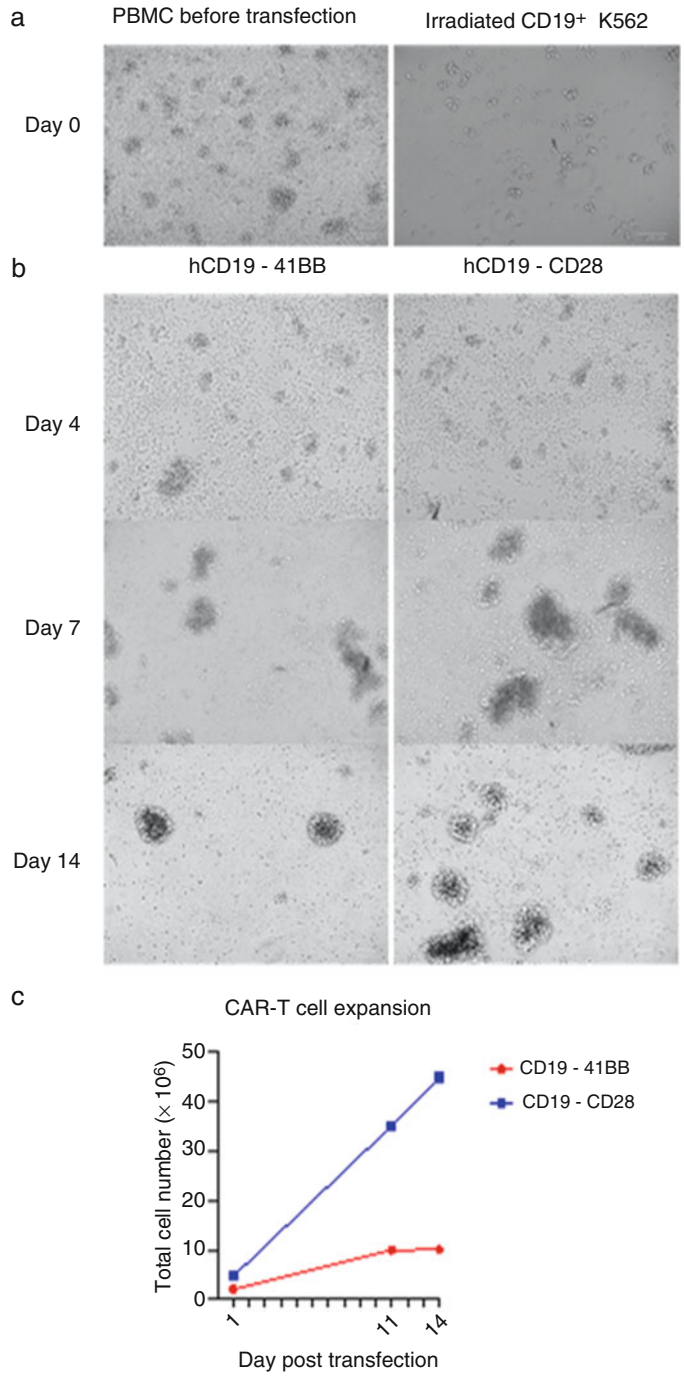

Fig. 3 Monitoring CAR-T cell expansion. (**a**, **b**) Sample microscope images of (**a**) day 0 PBMCs prior to transfection (left) and $CD19^+$ K562 cells (right) and (**b**) expanded PBMCs transfected with PiggyBac + pTpB-hCD19-41BB (left) or pTpB-hCD19-CD28 (right) at day 4, 7 and 14 post transfection. (**c**) Line chart depicting growth curves of CAR-T cells throughout 14 days of expansion

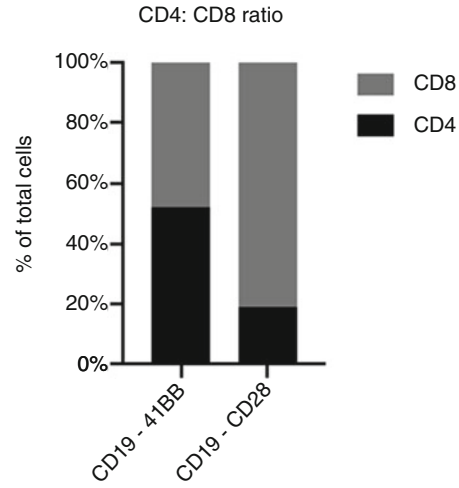

Fig. 4 Flow cytometry-based analysis of CAR-T cell phenotype showing the ratio of $CD4^+$ and $CD8^+$ T cells after 14 days of CAR-T cell expansion. $CD4^+$ and $CD8^+$ T cells shown as a percent of the total number of $CD3^+$ expanded CAR-T cells

5. Aspirate media and wash twice with PBS at 500 × g for 3 min at 4 °C.
6. Assess cell density and viability and resuspend cells in round-bottom tubes at 5×10^5 cells/tube.
7. Incubate cells in 100 μL of staining buffer containing BV421 mouse anti-human CD3 (5 μL/test), PE-Cy7 mouse anti-human CD4 (5 μL/test) and APC mouse anti-human CD8 (20 μL/test) in the dark for 30 min at 4 °C.
8. For single-stain controls, add one drop of UltraComp eBeads to 100 μL of staining buffer. Add the appropriate single stain to each of the tubes at the volumes listed above. Incubate in the dark for 30 min at 4 °C.
9. Wash twice with staining buffer.
10. Resuspend samples in 200 μL PBS and analyze samples using a CytoFLEX LX flow cytometer. Gate samples on $CD3^+$ cells (Fig. 4).

3.7 Generation of HEK293Tscan-CD19+ Cells

Transfection was performed by following the guidelines of the Neon Transfection System. Below is a brief outline of the method for transfection.

1. $HEK293^{HLA-}$ (*see* **Note 15**) cell density and viability were assessed.
2. Resuspend cells in a final solution containing 5×10^6 viable cells +5 ug of *PiggyBac* transposase DNA + 5 ug of pTpB-CMV-Tscan-IRES-hmbCD19 DNA in a total volume of 100 ul of resuspension buffer R.

3. Fill a Neon Electroporation Tube with 3 mL of electrolytic buffer E2 and insert tube into the neon pipette station.
4. Using the Neon Transfection System Pipette, fill a 100 μl neon tip with 100 μl of the cell/DNA mixture.
5. Transfer the pipette and tip containing the cell/DNA mixture into the electroporation tube ensuring there are no air bubbles (*see* **Note 10**).
6. Insert the neon pipette with the sample vertically into the neon tube until you hear a click sound.
7. Select 1100 volts (v), 20 pulse width (ms), and 2 pulses. Press start.
8. Once the program is completed, transfer the sample into a 6-well plate containing 3 mL of HEK-293 T media per well. Incubate transfected cells overnight at 37 °C with 5% CO_2.

3.8 Assessing the CAR-T Killing Capacity Using HEK293Tscan-CD19+ (Granzyme Attack Assay), CAR-T Cells and HEK293Tscan-CD19+ Cells Were Cocultured for 24 H to Assess the CAR-T Cell Killing Function

1. Assess cell density and viability of HEK293Tscan CD19+ cells. Plate HEK293Tscan CD19+ cells in a 6-well cell culture flat-bottom plate at 4×10^5 in HEK media.
2. Allow ~8 h for HEK293Tscan CD19+ cells to adhere to plate.
3. After HEK293Tscan have adhered to the plate bottom, aspirate media.
4. Locate an aliquot of previously cryopreserved CD19–41BB and CD19–CD28 CAR-T cells.
5. Thaw cells quickly in a 37 °C water bath (*see* **Note 13**).
6. Mix and add entire contents of the vial to 9 mL of T cell media and mix gently.
7. Centrifuge at 500 × g for 3 min at 4 °C.
8. Aspirate media and wash twice with PBS at 500 × g for 3 min at 4 °C.
9. Assess cell density and viability and resuspend cells in round-bottom tubes at 4×10^5 cells/mL in T cell media.
10. Add 4 mL of cell suspension on top of adherent HEK293Tscan-CD19+ resulting in a 4:1 ratio of CAR-T cells: CD19+ HEK293Tscan-CD19+ cells (*see* **Note 14**).
11. Prepare negative control well containing only "parental" untransfected (CD19-) HEK293Tscan cells with 4:1 ratio of CAR-T cells: HEK293Tscan.
12. Incubate the cocultured cells for 24 h at 37 °C with 5% CO_2.

3.9 CAR-T Cell Functional Analysis

After the HEK293Tscan and CAR-T cell coculture, CAR-T cell functionality was assessed by measuring granzyme activity (APC/Cy7) in HEK293Tscan-CD19+ reporter cells.

1. Following the 24-h incubation, collect the media containing CAR-T cells.

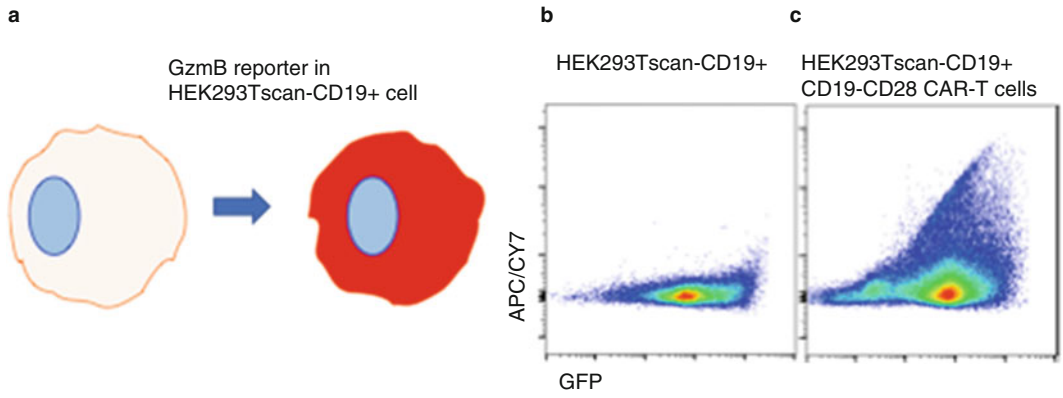

Fig. 5 Flow cytometry-based analysis of CAR-T cell function. (**a**) Schematic depicting the granzyme B reporter in the Tscan target cells, indicating activation of an APC/Cy7, indicating granzyme-mediated activity in the HEK293T-CD19^{+} target cells. (**b**–**c**) Representative FAC plots of granzyme B activity reported by fluorescence in Tscan cells (**b**) alone or (**c**) cocultured in the presence of CD19–CD28 CAR-T cells

2. Rinse wells with PBS.
3. Trypsinize the HEK293Tscan with 0.25% trypsin–EDTA and collect HEK293Tscan.
4. Spin at 500 × g for 3 min at 4 °C.
5. Wash twice with PBS at 500 × g for 3 min at 4 °C.
6. Resuspend samples in 200 μL PBS and analyze samples using a CytoFLEX LX flow cytometer gating on GFP+ (HEK293Tscan cells) and APC/Cy7 (granzyme attack reporter) (Fig. 5).

4 Notes

1. The 4-1BB CAR construct was designed to be similar to the clinical JCAR14–17 [19] briefly, FM63 scFv linked to a short IgG1 hinge domain and a CD8-derived transmembrane domain followed by a 41BB costimulation domain and a CD3zeta c terminus.
2. The CD28 CAR construct was synthesized by Genewiz (South Plainfield, NJ) and designed to be similar to KTE-C19 [19]; briefly the FM63 scFv is linked to the CD28 transmembrane domain and signaling domain with CD3z c-terminus.
3. The pTpB-CMV-hmbCD19 *PiggyBac* transposon construct encoding a membrane-bound human CD19 driven by the CMV promoter was synthesized by Vector builder.
4. All plasmids are resuspended in distilled deionized water (Milli-Q Biocel, Millipore, USA).
5. It is important that the plasmid preparation system contains an endotoxin removal step.

6. When possible, freshly isolated PBMCs should be used. If using thawed PBMCs, 24-h recovery in T cell media + 10 ng/mL recombinant IL-7 (4.4×10^5 IU/ug) is essential prior to transfection.
7. Lympholyte® cell separation media should be <6 months old and stored according to manufacturer's suggestions.
8. To avoid disturbing the gradient by sudden braking, shut the brake off and allow the centrifuge to run until the rotor stops by itself.
9. Determine the cell number and viability using the trypan blue dye exclusion method. Cell viability should be >90% before proceeding with transfection.
10. To prevent air bubbles, a cell/DNA mixture containing 1.1× the reaction volume may be prepared to ensure transfer to the cuvette/neon tip without the presence of bubbles.
11. When expanding CAR+ T cells, it is important that the feeder cell line (in this case CD19+ K562 cells) does not express HLA I or II to avoid nonspecific alloreactive expansion of unmodified T cells.
12. Upon initial freezing of cells at −80 °C, place cells into a freezing container (Nalgene ® Mr. Frosty) in order to provide the critical and repeatable 1 °C per minute cooling rate for successful cryopreservation of cells.
13. When performing killing assay, it may be helpful to thaw the cells and rest overnight with 50 IU/mL rIL-2 before running killing assay.
14. When conducting killing assays like the granzyme reporter assay described here, it is helpful to minimize the total volume of media to maximize the interaction between CAR-T cells and target cells.
15. It is important that the target cells used for Tscan-granzyme reporter assay be negative for the targeted surface antigen (in this case CD19) and either be HLA matched to the donor or be HLA-null to prevent allogeneic granzyme attack. We find that HEK293 cells which have all HLA A/B/C knockout work well for this application, but it is possible that other cell types could be deployed similarly. The hmbCD19 contained in the pTpB-EF1a-Tscan-hmbCD19 can be replaced with alternative CAR-specific antigens when exploring the function of other CARs.

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Chapter 15

Redirecting Human Conventional and Regulatory T Cells Using Chimeric Antigen Receptors

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Abstract

The adaptive immune system exhibits exquisite specificity and memory and is involved in virtually every process in the human body. Redirecting adaptive immune cells, in particular T cells, to desired targets has the potential to lead to the creation of powerful cell-based therapies for a wide range of maladies. While conventional effector T cells (Teff) would be targeted towards cells to be eliminated, such as cancer cells, immunosuppressive regulatory T cells (Treg) would be directed towards tissues to be protected, such as transplanted organs. Chimeric antigen receptors (CARs) are designer molecules comprising an extracellular recognition domain and an intracellular signaling domain that drives full T cell activation directly downstream of target binding. Here, we describe procedures to generate and evaluate human CAR $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and $CD4^+FOXP3^+$ regulatory T cells.

Key words T cell, Regulatory T cell, Chimeric antigen receptor, Synthetic immunology, Immune assay

1 Introduction

Our adaptive immune system has evolved to specifically recognize a virtually infinite variety of molecules (antigens). This allows it to distinguish pathogens (nonself) and cancerous cells (altered self) from our own tissues (self) and symbiotic microbiota (extended self). T cells are at the heart of this recognition system, with each T cell bearing a T cell receptor (TCR) specific for a unique peptide epitope presented on a major histocompatibility complex (MHC) molecule. Cytotoxic $CD8^+$ T cells are restricted to MHC class I molecules, whereas helper $CD4^+$ T cells are restricted to MHC class II molecules [1]. It is thus highly desirable to identify, isolate, and expand antigen-specific T cells for therapy. Yet, these are vanish-

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ingly rare. Moreover, disease epitopes are often unknown, and it remains extremely challenging to predict TCR–epitope pairs based on amino acid sequence alone [2].

Synthetic biology can be used to impart a desired specificity to T cells, partly circumventing the hurdles indicated above and greatly expanding what targets can be pursued using T cell-based therapies. Chimeric antigen receptors (CARs) are synthetic immune receptors consisting of an extracellular antibody-based antigen-binding domain, known as a single-chain fragment variable (scFv), and an intracellular signaling domain. By combining T cell signal 1 (TCR recognition, typically CD3zeta) and signal 2 (co-stimulation, typically CD28) in its endomain, second-generation CARs elicit potent T cell activation directly downstream of antigen recognition [3].

CAR T cells have changed the landscape of cancer therapy, inducing high rates of remission in previously incurable cancers. Currently, there are five CAR T cell therapies for cancer approved by the US Food and Drug Administration (FDA). Nevertheless, most of these are CAR T cells specific for CD19, a B cell-derived leukemia marker, and all of these are for liquid tumors. Solid tumors remain refractory to CAR T cell therapy and the search for effective targets is object of intense investigation [4]. Still, the success of CD19 CAR T cell therapies has prompted researchers to look beyond cancer, creating CAR T cells that target other undesirable cells for destruction, such as senescent cells [5] and activated fibroblasts during acute cardiac injury [6]. Moreover, it has kindled interest in engineering other immune cells with CARs, including natural killer (NK) cells [7], macrophages [8], and regulatory T cells (Tregs) [9]. A subset of $CD4^+$ T cells dedicated to suppressing immune responses, $CD4^+FOXP3^+$ Tregs are essential to maintain immune tolerance and homeostasis. Manipulating Treg specificity with CAR technology offers the opportunity to modulate the immune system with antigen specificity in organ transplant rejection, autoimmunity, and inflammation [10]. Hence, CARs can be used both to redirect $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells to increase immunity in the context of cancer and to redirect $CD4^+FOXP3^+$ Tregs to decrease immunity in the context of organ transplant rejection and autoimmune disease (Fig. 1a).

Altogether, much ongoing research is dedicated to creating and testing new CAR targets and CAR designs. CARs can be used to target T cells to specific tissues and disease states independently of endogenous antigens recognized by resident lymphocytes, which have often revealed difficult to identify, especially in humans. More broadly, the CAR platform has the power to help dissect how specificity, affinity, and signaling modulate the function of different T cell subsets in tolerance and immunity, ultimately informing the design and development of new engineered immune cell-based therapies for a variety of disorders. Given the increasing and

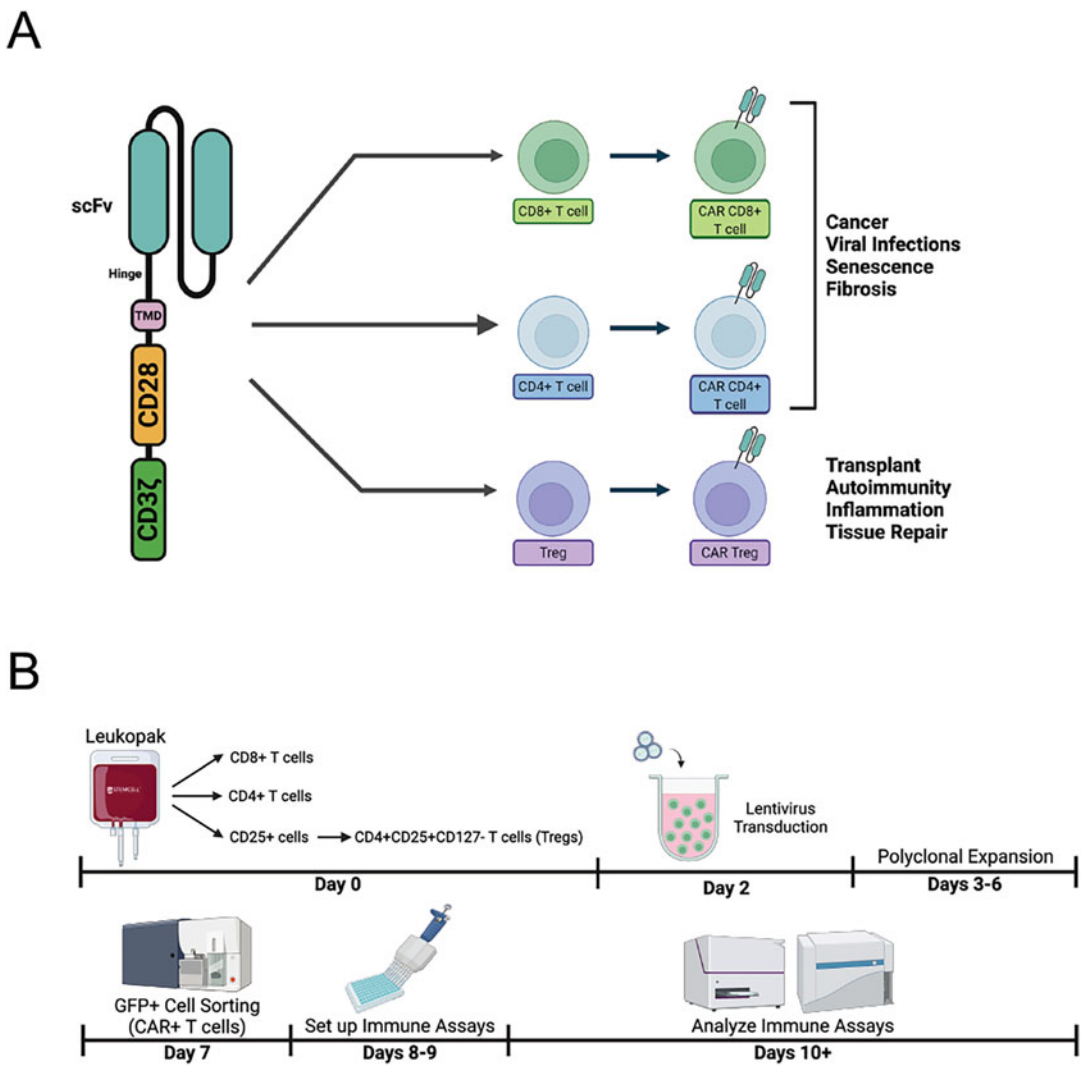

Fig. 1 Redirecting T cells using chimeric antigen receptors**.** (**a**) A chimeric antigen receptor (CAR) comprises an antigen recognition domain, typically a single-chain fragment variable (scFv). This domain is connected to an intracellular signaling domain, typically a tandem CD28-CD3zeta domain, by a hinge and a transmembrane domain. Introducing a CAR in $CD4^+$ T cells and/or $CD8^+$ T cells can be used to combat cancer, viral infections, senescence, and fibrosis, whereas introducing a CAR in regulatory T cells (Tregs) can be used to prevent transplant rejection, treat autoimmunity and excessive inflammation, as well as aid tissue repair. (**b**) The workflow of generating human CAR T cells involves purifying and activating T cell subsets from a human peripheral blood product, e.g., a leukopak (day 0); transducing activated T cells with CAR lentivirus (day 2); polyclonally expanding transduced cells (days 3–6); sorting CAR^+ T cells (e.g., by sorting cells expressing a CAR reporter gene, such as GFP) (day 7); setting up immune assays, namely, cytotoxicity or suppression (days 8–9); and finally analyzing such immune assays (day 10 and beyond)

broadening importance of engineering CAR T cell subsets, we provide detailed protocols to create and test human CAR $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and $CD4^+FOXP3^+$ regulatory T cells.

2 Materials

2.1 T Cell Isolation

- 1/10 Leukopak (STEMCELL Technologies #200-0092).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- RPMI 1640 medium, no glutamine (Gibco #11875093).
- Fetal bovine serum (FBS) (Gibco #26140079).
- Penicillin–streptomycin solution (Gibco #15140122).
- GlutaMAX (Gibco #35050061).
- Sodium pyruvate (Gibco #11360070).
- Nonessential amino acid (NEAA) solution (Gibco #11140050).
- 1 M HEPES (Gibco #15630080).
- Ammonium chloride solution (STEMCELL Technologies #07850).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- 0.5 M EDTA, pH 8.0 (Gibco #15575020).
- EasySep magnet (STEMCELL Technologies #18000).
- EasySep Human $CD8^+$ T Cell Enrichment Kit (STEMCELL Technologies #19053).
- EasySep Human $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies #19052).
- Easy 50 EasySep magnet (STEMCELL Technologies #18002).
- EasySep Human Pan-CD25 Positive Selection and Depletion Kit (STEMCELL Technologies #17861).
- Anti-human CD4 FITC (clone SK3, Biolegend #344604).
- Anti-human CD25 APC (clone BC96, Biolegend #302610).
- Anti-human CD127 PE (clone hIL-7R-M21, BD Biosciences #557938).
- Anti-human CD8 PerCP (clone SK1, Biolegend #344707).
- Flow cytometer (e.g., Beckman CytoFLEX).

- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).

2.2 Lentivirus Production

- HEK293T cells (ATCC #CRL-3216).
- Jurkat cells (ATCC #TIB-152).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- RPMI10 complete medium (*see* Subheading 3.1).
- DMEM, high glucose, no glutamine medium (Gibco #11960044).
- Fetal bovine serum (FBS) (Gibco #26140079).
- GlutaMAX (Gibco #35050061).
- Sodium pyruvate (Gibco #11360070).
- Nonessential amino acid (NEAA) solution (Gibco #11140050).
- Trypsin–EDTA (0.25%) (Gibco #25200056).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Chloroquine diphosphate (Fisher Scientific #AAJ6445914).
- Polyethylenimine (PEI), linear (Sigma-Aldrich #919012-100MG).
- Nuclease-free water (New England Biolabs #B1500L).
- Opti-MEM reduced serum medium (Gibco #31985070).
- Packaging DNA plasmid (e.g., psPAX2, Addgene #12260).
- Envelope DNA plasmid (e.g., VSV-G, Addgene #8454).
- Transfer DNA plasmid (e.g., CD19CAR-2A-GFP, Addgene #135991).
- ViralBoost (ALSTEM #VB100).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.3 T Cell Transduction

- T cells isolated and activated in Subheading 3.1.
- Lentivirus produced and titrated in Subheading 3.2.
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- RPMI10 complete medium (*see* Subheading 3.1).

- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.4 CAR+ T Cell Sorting

- T cells transduced in Subheading 3.3.
- RPMI10 complete medium (*see* Subheading 3.1).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- FACS sorter (e.g., BD FACS Aria II Cell Sorter).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200-02).

2.5 Immune Assays

2.5.1 Activation

- CAR^+ T cells sorted in Subheading 3.4.
- K562 cells (ATCC #CCL-243).
- CAR target-expressing K562 cells (e.g., CD19-K562).
- Cesium-137 irradiator.
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200-02).
- RPMI10 complete medium (*see* Subheading 3.1).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Anti-human CD4 PE/Cy7 (clone SK3, Biolegend #344612).
- Anti-human CD8 PerCP (clone SK1, Biolegend #344708).
- Ghost BV510 viability dye (TONBO #13-0870-T100).
- Anti-human CD71 PE (clone SK1, Biolegend #334106).
- Anti-human CD25 APC (clone BC96, Biolegend #302610).
- Flow cytometer (e.g., Beckman CytoFLEX).
- Human IFN-γ Quantikine ELISA kit (R&D Systems #DIF50C).

- Human IL-10 Quantikine ELISA kit (R&D Systems #D1000B).
- Microplate reader (e.g., Molecular Devices SpectraMax).

2.5.2 Cytotoxicity

- CAR^+ T cells sorted in Subheading 3.4.
- K562 cells (ATCC #CCL-243).
- CAR target-expressing K562 cells (e.g., CD19-K562).
- RPMI10 complete medium (*see* Subheading 3.1).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200-02).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- CyQUANT LDH Cytotoxicity Assay (Thermo Fisher #C20300).
- Microplate reader (e.g., Molecular Devices SpectraMax).

2.5.3 Suppression

- CAR^+ Treg cells sorted in Subheading 3.4.
- CAR target-expressing K562 cells (e.g., CD19-K562).
- Cesium-137 irradiator.
- Unmodified $CD4^+$ T cells from Subheading 3.1.
- Unmodified $CD8^+$ T cells from Subheading 3.1.
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- RPMI10 complete medium (*see* Subheading 3.1).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- CellTrace Violet Proliferation Kit (Thermo Fisher #C34571).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Anti-human CD4 PE/Cy7 (clone SK3, Biolegend #344612).
- Anti-human CD8 PerCP (clone SK1, Biolegend #344708).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.5.4 Expansion

- CAR^+ T cells sorted in Subheading 3.4.
- CAR target-expressing K562 cells (e.g., CD19-K562).
- Cesium-137 irradiator.
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- RPMI10 complete medium (*see* Subheading 3.1).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Anti-human CD4 PE/Cy7 (clone SK3, Biolegend #344612).
- Anti-human CD8 PerCP (clone SK1, Biolegend #344708).
- Ghost BV510 viability dye (TONBO #13-0870-T100).
- Precision Count Beads (Biolegend #424902).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.5.5 Exhaustion

- CAR^+ T cells sorted in Subheading 3.4.
- CAR target-expressing K562 cells (e.g., CD19-K562).
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- RPMI10 complete medium (*see* Subheading 3.1).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Anti-human CD4 Alexa Fluor 700 (clone SK3, Biolegend #344622).
- Anti-human CD8 APC/Cy7 (clone SK1, Biolegend #344714).

- Anti-human PD-1 PerCP (clone EH12.2H7, Biolegend #329938).
- Ghost BV510 viability dye (TONBO #13-0870-T100).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.5.6 Stability

- CAR^+ Treg cells sorted in Subheading 3.4.
- CAR target-expressing K562 cells (e.g., CD19-K562).
- Cesium-137 irradiator.
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- RPMI10 complete medium (*see* Subheading 3.1).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).
- DynaMag-15 magnet (Thermo Fisher #12301D).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- eBioscience Foxp3 transcription factor staining buffer set (Thermo Fisher #00-5523-00).
- Anti-human CD4 PE/Cy7 (clone SK3, Biolegend #344612).
- Anti-human CD25 APC (clone BC96, Biolegend #302610).
- Ghost BV510 viability dye (TONBO #13-0870-T100).
- Anti-human FOXP3 eFluor 450 (clone PCH101, Thermo Fisher #48-4776-42).
- Anti-human HELIOS PE (clone 22F6, Biolegend #137216).
- Anti-human CTLA-4 PerCP-e710 (clone 14D3, Thermo Fisher #46-1529-42).
- Flow cytometer (e.g., Beckman CytoFLEX).

3 Methods

3.1 T Cell Isolation

Prior to generating chimeric antigen receptor (CAR) T cells via viral transduction of human T cells and testing them in immune assays, T cells must first be isolated from peripheral blood mononuclear cells (PBMCs) obtained from a leukopak (Fig. 1b). Subsequently, these PBMCs are magnetic enrichment with various kits to isolate different T cell subsets, such as $CD4^+$ (Fig. 2a) and $CD8^+$ T cells

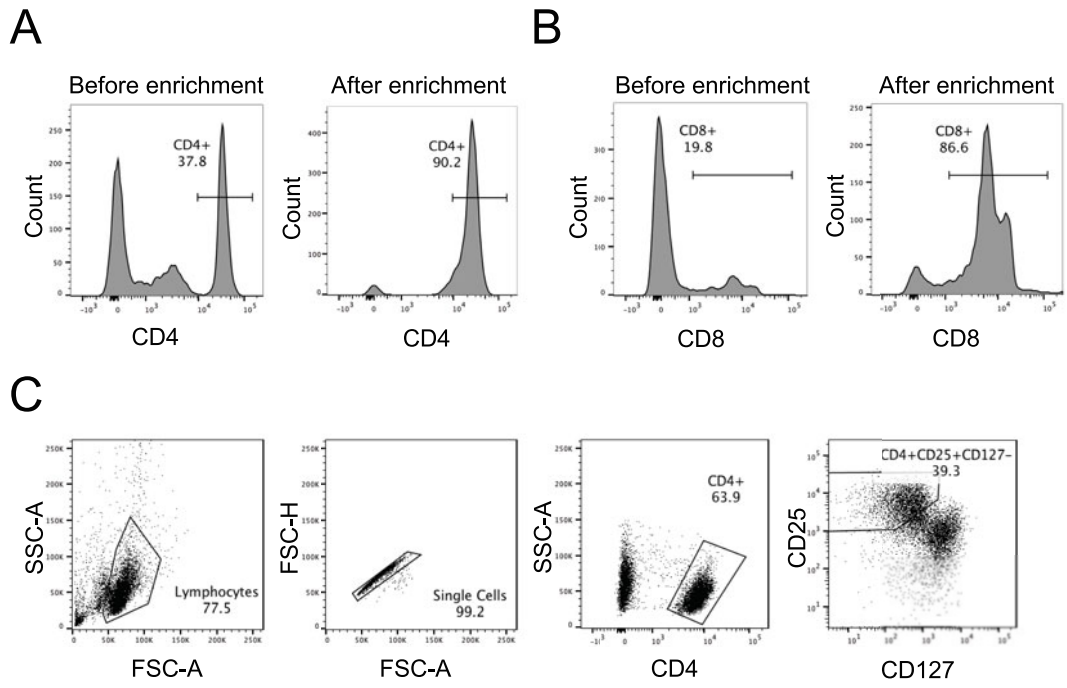

Fig. 2 Purifying T cell subsets from human peripheral blood. (**a**) Purity of human $CD4^+$ T cells after magnetic negative selection enrichment from a leukopak, as assessed by flow cytometry with anti-human CD4 PE/Cy7. (**b**) Purity of human CD8+ T cells after magnetic negative selection enrichment from a leukopak, as assessed by flow cytometry with anti-human CD8 PerCP. (**c**) Fluorescence-assisted cell sorting (FACS) gating strategy to purify human $CD4^+CD25^+CD127^-$ Treg cells after magnetic positive selection enrichment of $CD25^+$ cells from a leukopak, followed by staining with anti-human CD4 FITC, anti-human CD25 APC, and anti-human CD127 PE

(Fig. 2b). For the isolation of regulatory T cells (Tregs), their rarity (ca. 1% of PBMCs or 5% of $CD4^+$ T cells) requires a magnetic enrichment step to obtain a cell population enriched in $CD25^+$ cells, followed by a fluorescence-assisted cell sorting (FACS) step to isolate $CD4^+CD25^+CD127^-$ Tregs with over 95% purity (Fig. 2c).

3.1.1 Leukopak Processing

1. Dilute the leukopak in an equivalent volume of DPBS +2% FBS. Mix by pipetting up and down slowly.
2. Count cells by diluting them first 1:100 with DPBS (10 µl of cell suspension +90 µl of DPBS and then 10 µl of cells diluted 1:10 + 90 µl of DPBS) and then 1:1 with Trypan Blue solution (10 µl of cells diluted 1:100 + 10 ul Trypan Blue). If using an automated cell counter, make sure to adjust cell count to this 200-fold cell dilution by multiplying the result by 100, as most cell counters assume a 1:1 dilution.
3. Centrifuge the cells at 300 *g* for 10 min at room temperature (RT) with the brake *on*.

4. Aspirate the supernatant, leaving some volume behind to avoid disturbing the cell pellet.
5. Resuspend the cell pellet, add 2 mL of DPBS +2% FBS, and pipette up and down.
6. Add 8 mL of ammonium chloride solution to the cell suspension (4:1 ratio). Pipette up and down.
7. Incubate on ice for 15 min.
8. Centrifuge at 500 *g* for 10 min at RT with the brake *on*.
9. Aspirate the supernatant.
10. Wash by adding 30 mL DPBS +2% FBS.
11. Centrifuge at 150 *g* for 10 min at RT with the brake *off*.
12. Aspirate the supernatant and resuspend the cell pellet in 30 mL DPBS +2% FBS.
13. Count cells by diluting them first 1:100 with DPBS (10 μl of cell suspension +90 μl of DPBS and then 10 μl of cells diluted 1:10 + 90 μl of DPBS) and then 1:1 with Trypan Blue solution (10 μl of cells diluted 1:100 + 10ul Trypan Blue). If using an automated cell counter, make sure to adjust cell count to this 200-fold cell dilution by multiplying the result by 100, as most cell counters assume a 1:1 dilution.

3.1.2 $CD4^+$ T Cell Enrichment (Negative Selection)

1. Collect 15×10^6 PBMCs and centrifuge at 500 *g* for 5 min at RT. The starting cell number will depend on your specific needs.
2. Resuspend the cells in a Cell Separation Buffer (DPBS +10 mM EDTA +2% FBS) at 50×10^6 cells/mL.
3. Follow EasySep Human $CD4^+$ T Cell Enrichment Kit protocol.
4. Count cells in a 1:1 ratio with Trypan Blue.
5. Assess $CD4^+$ T cell purity using flow cytometry (Fig. 2a).

3.1.3 $CD8^+$ T Cell Enrichment (Negative Selection)

1. Collect 50×10^6 PBMCs and centrifuge at 500 *g* for 5 min at RT. The starting cell number will depend on your specific needs.
2. Resuspend the cells in Cell Separation Buffer (DPBS +10 mM EDTA +2% FBS) at 50×10^6 cells/mL.
3. Follow EasySep Human $CD8^+$ T Cell Enrichment Kit protocol.
4. Count cells in a 1:1 ratio with Trypan Blue.
5. Assess $CD8^+$ T cell purity using flow cytometry (Fig. 2b).

3.1.4 $CD25^+$ Cell Enrichment (Positive Selection)

1. Collect 109 PBMCs and centrifuge at 500 g for 5 min at RT. The starting cell number will depend on your specific needs.
2. Resuspend the cells in Cell Separation Buffer (DPBS +10 mM EDTA +2% FBS) at 108 cells/mL.
3. Follow EasySep Human Pan-CD25 Positive Selection and Depletion Kit protocol.
4. Count $CD25^+$ cells in a 1:1 ratio with Trypan Blue.

3.1.5 $CD4^+CD25^+CD127^-$ Treg Sorting (FACS)

1. Centrifuge $CD25^+$ cells obtained in Subheading 3.1.4 at 500 *g* for 5 min at RT.
2. Resuspend the cells in 200 μL of DPBS.
3. Add the following antibodies: anti-human CD4 FITC, anti-human CD25 APC, anti-human CD127 PE at 1 μL of antibody per 10^6 cells. Make sure to use the antibody clones indicated in the materials Subheading (2.1), especially CD25 (clone BC96), as the $CD25^+$ cell fraction will have some of the CD25 epitopes blocked by the antibodies used in the EasySep Human Pan-CD25 Positive Selection and Depletion Kit.
4. Incubate at 4 °C for 30 min in the dark.
5. Wash by adding 10 mL of DPBS, and then centrifuge at 500 *g* for 5 min at RT.
6. Resuspend cells at 15×10^6 cells/mL in DPBS.
7. Filter cell suspension through a 40-μm filter cap into a FACS tube and put on ice.
8. Prepare collection tubes for sorting by adding 3 mL RPMI10 complete medium (*see* **Note 1**) per 15-mL conical tube and put on ice. Collection tube type and complete medium volume will depend on the specific cell sorting instrument used.
9. Sort $CD4^+CD25^+CD127^-$ cells using fluorescence assisted cell sorting (FACS) (Fig. 2c).
10. Count the cells post-sort in a 1:1 ratio with Trypan Blue.

3.1.6 T Cell Activation

1. After T cell subset purification and counting, activate T cells in a 24-well plate with Human T-Activator CD3/28 for T Cell Expansion and Activation Dynabeads and recombinant human IL-2 (rhIL-2) in RPMI10 complete medium.
2. Add 25 μL of anti-CD3/CD28 Dynabeads for every 10^6 T cells obtained for a 1:1 ratio of Dynabeads to T cells.
3. Add recombinant human IL-2:
   (a) Add 1000 IU/ml of rhIL-2 to Treg cells.
   (b) Add 100 IU/ml of rhIL-2 to $CD4^+$ T cells.
   (c) Add 300 IU/ml of rhIL-2 to $CD8^+$ T cells.

4. Plate T cells at 5×10^5 cells per well of a 24-well plate in 1 ml RPMI10 complete medium and rhIL-2 and put in a 37 °C 5% CO_2 tissue culture incubator (*see* **Note 2**, Fig. 3).

3.2 Lentivirus Production

Second-generation lentivirus production employs transfection of human embryonic kidney 293 T (HEK293T) cells with DNA plasmids encoding lentiviral packaging proteins, lentiviral envelope proteins, and the construct of interest, in this case a CAR. HEK293T cells are ideal to produce lentivirus as they are easily maintained in culture and transfected with very high efficiencies. Once the plasmid DNA is taken up by the cells, the HEK293T cellular machinery produces lentivirus particles containing the CAR gene and releases them into the culture medium (Fig. 4a). The medium containing the lentivirus particles is then titrated in Jurkat cells and stored in aliquots at −80 °C for subsequent transduction of primary human T cells.

Day 0

1. Count HEK293T cells with Trypan Blue and seed at a density of 3.8×10^6 cells per 10-cm dish in DMEM10 complete medium. Cells should be expanded ahead of time and then split into the desired number of plates. The timeline of transfection is as follows (Table 1):
2. Prepare stock solutions of chloroquine diphosphate and polyethylenimine (PEI) for transfection.
3. Dissolve 0.129 g of chloroquine diphosphate salt in 10 mL of sterile nuclease-free water to prepare a 25 mM chloroquine stock solution.
4. Sterilize the 25 mM chloroquine stock solution using a 10-mL syringe and 0.22-μm filter. Aliquot and store at −20 °C as needed.
5. Dissolve 0.1 g of PEI in 90 mL of ultrapure water (via Milli-Q Water Purification System) in a 150-mL autoclaved beaker and stir using a magnetic stir bar/stir plate to prepare a 1 mg/mL PEI stock solution.
6. Adjust pH to 7.0 by progressively adding small volumes of either HCl (to decrease pH) or NaOH (to increase pH) while measuring the pH using a pH meter.
7. Sterilize the 1 mg/mL PEI pH 7.0 stock solution through a 0.22-μm filter using a 10-mL syringe. Aliquot and store at −80 °C as needed.
8. Test chloroquine and PEI solutions on 6×10^5 of HEK293T cells in a 6-well with 1 μg of DNA to ensure they result in sufficient transfection efficiency prior to aliquoting/freezing

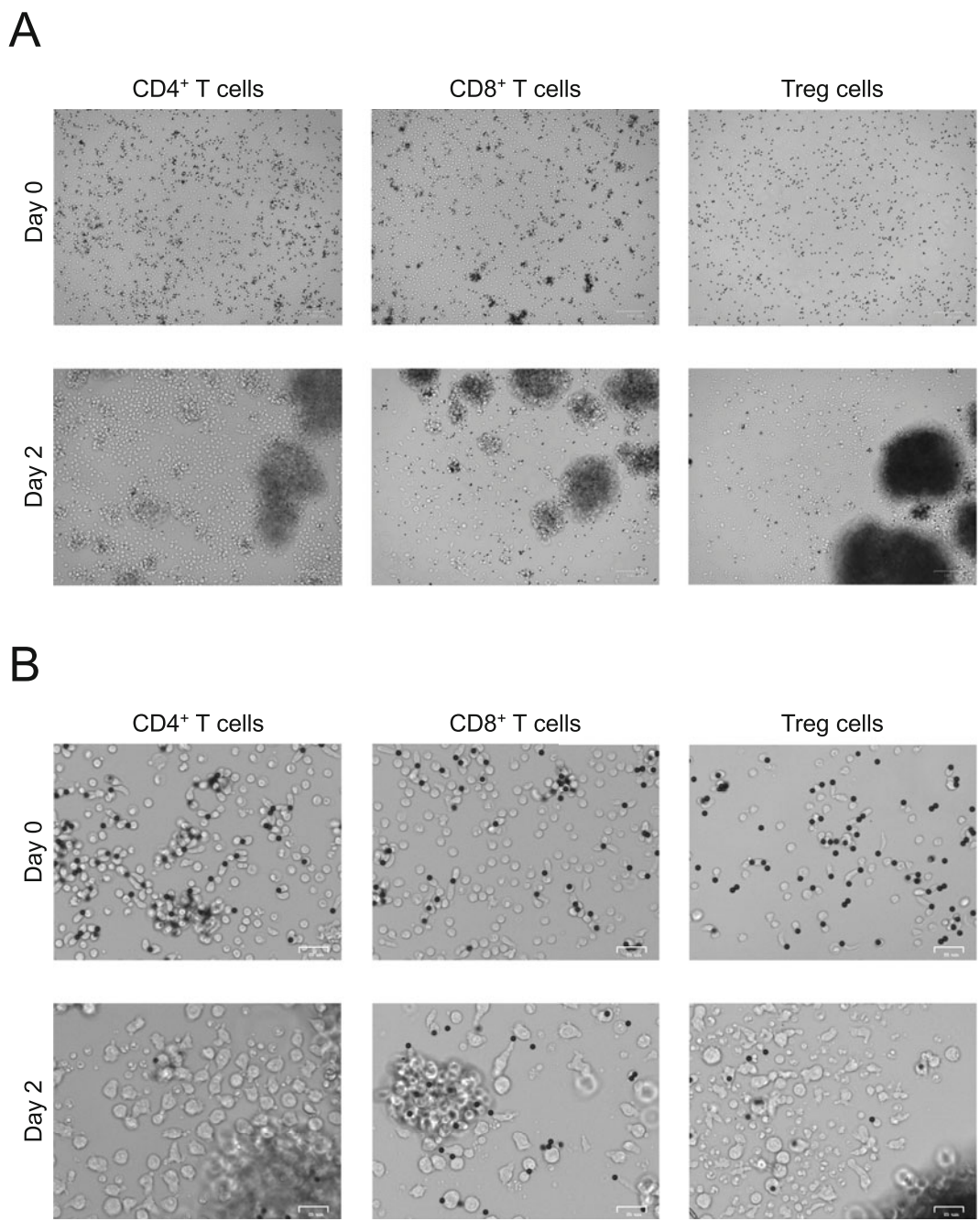

Fig. 3 Activation of purified human T cell subsets. (**a**) Anti-CD3/CD28 bead-mediated activation (1:1 bead to cell ratio) of human $CD4^+$ T cells, $CD8^+$ T cells, and Treg cells for 48 h. Images at 5× magnification. (**b**) Anti-CD3/CD28 bead-mediated activation of human $CD4^+$ T cells, $CD8^+$ T cells, and Treg cells for 48 h. Images at 20× magnification

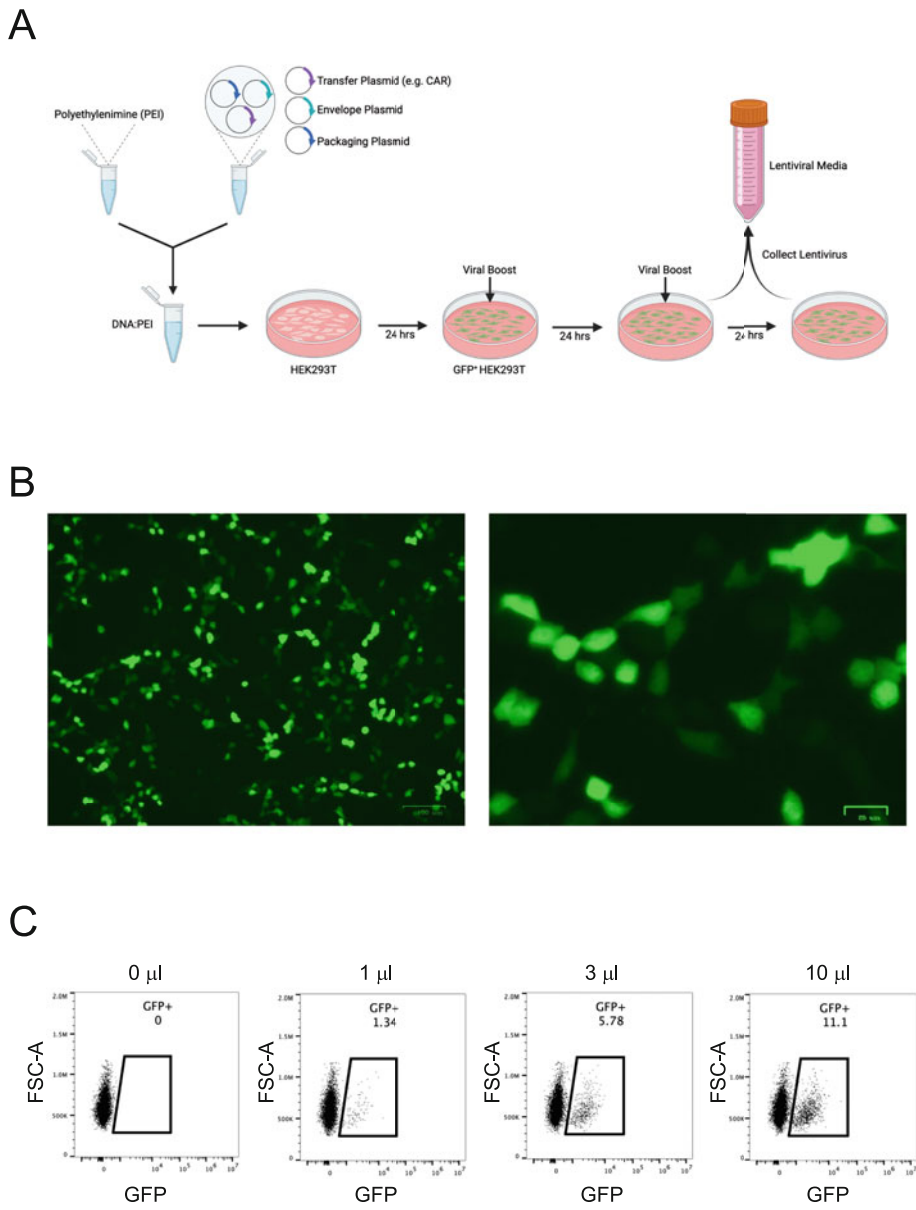

Fig. 4 Lentivirus production and titration. (**a**) The workflow for lentivirus production involves transfecting chloroquine-treated HEK293T cells with transfer plasmid (CAR), envelope plasmid, and packaging plasmid using polyethylenimine (PEI). One day later, the medium is replaced with fresh medium containing ViralBoost. On the third and fourth days, lentivirus-containing medium is collected into conical tubes and stored at 4 °C. (**b**) GFP expression by HEK293T cells 18 h after transfection with CD19CAR-2A-GFP accompanied by envelope plasmid and packaging plasmid, imaged at 5× (left) and 20× (right) magnification. (**c**) Titration of CD19CAR-2A-GFP lentivirus particle-containing medium in Jurkat cells

Table 1
Timeline of HEK293T transfection for lentivirus production

Monday (D0)	Tuesday (D1)	Wednesday (D2)	Thursday (D3)	Friday (D4)
Seed cells at 3.8×10^6 cells per 10-cm dish	Add chloroquine incubate 5 h Transfect with DNA/PEI complexes	Change medium Add ViralBoost	Collect viral medium Add fresh medium + ViralBoost	Collect viral medium Transduce Jurkat cells for virus titration

Table 2
Plasmid DNA concentrations used in second-generation CAR lentivirus production

Plasmid	DNA amount (μg)	Concentration (ng/μL)	Volume to add (μL)
Packaging plasmid (psPAX2)	6.66 μg		
Envelope plasmid (pCMV-VSV-G)	3.33 μg		
Transfer plasmid (pSLCAR-CD19-28z)	10 μg		

the solutions. The ideal PEI/DNA ratio will be PEI batch dependent. In our hands, a 2:1 PEI/DNA mass ratio yielded the best results.

Day 1

1. Aspirate exhausted medium.
2. Add 10 mL fresh DMEM10 complete medium with 25 μM chloroquine (10 μL of 25 mM chloroquine stock solution in 10 mL medium).
3. Incubate the plates at 37 °C 10% CO_2 in tissue culture incubator for 3–5 h.
4. Thaw DNA plasmids and PEI stock solution aliquots on ice.
5. Calculate the volume needed of each DNA plasmid using a table as below (Table 2). Fill out concentration and, subsequently, volume to add according to the specific plasmid DNA concentrations of the Maxiprep DNA batches in use.
6. Dilute the required volumes of the three DNA plasmids needed per construct (total of 20 μg of DNA) in 500 μl Opti-MEM in a 1.5-mL microcentrifuge tube.

7. Dilute 40 μl of 1 mg/mL PEI in 500 μl Opti-MEM in a separate 1.5-mL microcentrifuge tube (for a 2:1 PEI/DNA ratio).
8. Close both tubes and gently flick to mix contents.
9. Briefly spin down both tubes to ensure contents are in the bottom of the tube.
10. Slowly add the diluted PEI to the diluted DNA tube dropwise.
11. Gently flick the combined tube to mix contents. *Do not* vortex to prevent shearing of the plasmid DNA.
12. Incubate the tube for 15 min at RT.
13. Retrieve the 10-cm plate with HEK293T cells to be transfected from the incubator.
14. Add PEI/DNA complexes dropwise to the HEK293T cells, covering the plate.
15. Place the transfected HEK293T cell plate back in the tissue culture incubator.

Day 2

1. Twenty-four hours after transfection, use the ZOE fluorescence cell imager to confirm transfection prior to changing medium. In this specific example, HEK293T will be GFP^+ if successfully transfected (Fig. 4b).
2. Aspirate exhausted medium and add 10 mL DMEM10 complete medium (*see* **Note 3**) + 20 μL ViralBoost to maximize viral production.
3. Place the plate back in the tissue culture incubator.

Day 3

1. Collect lentivirus-containing medium from plate into a 50-mL conical tube. Forty-eight hours post-transfection is the peak of lentivirus production.
2. Add 10 mL DMEM10 complete medium +20 μL ViralBoost to maximize viral production.
3. Place the plate back in the tissue culture incubator.
4. Store the collected lentivirus-containing medium at 4 °C.

Day 4

1. Collect lentivirus-containing medium into a 50-mL conical tube from the previous day.
2. Discard the HEK293T cell plate.
3. Store the collected lentivirus-containing medium at 4 °C.

Titration

1. Count Jurkat cells with Trypan Blue and seed at a density of 10^4 cells per well of a 96-well round-bottom plate in 200 μl RPMI10 complete medium.
2. Add 10, 3, 2, or 1 μL lentivirus-containing medium per well with 10^4 Jurkat cells. Make sure to leave wells with untransduced Jurkat cells as negative controls.
3. Place the plate with transduced Jurkat cells in tissue culture incubator for 72 h.
4. Harvest cells and analyze via flow cytometry to measure transduction efficiency. In this specific case, determine % GFP^+ cells in each condition (Fig. 4c).
5. Use transduction efficiencies with different lentivirus-containing medium volumes to calculate the lentivirus titer, i.e., the number of lentivirus particles per volume of lentivirus-containing medium.
6. Perform a linear regression and extrapolate to determine volume required to transduce a desired number of T cells.
7. Centrifuge tubes with lentivirus-containing medium at 500 *g* for 5 min at 4 °C.
8. Carefully transfer the lentivirus-containing supernatant to fresh conical tubes. This step eliminates cellular debris.
9. Aliquot lentivirus-containing medium and store at −80 °C as needed. We transduce multiples of 2.5×10^5 T cells with CAR lentivirus for immune assays and hence make aliquots with the number of lentivirus particles needed to transduce 2.5×10^5 Jurkat cells.

3.3 T Cell Transduction

1. Count T cells with Trypan Blue.
2. Centrifuge cells at 500 *g* for 5 min at room temperature (RT).
3. Resuspend cells in RPMI10 complete medium at 1.25×10^6 cells/mL.
4. Thaw lentivirus aliquots on ice.
5. Add lentivirus aliquots to 2.5×10^5 T cells in 200 μl in a 1.5-mL microcentrifuge tube. $CD4^+$ and $CD8^+$ T cells are harder to transduce than Treg cells, so add 1 lentivirus aliquot per 2.5×10^5 Treg cells and 2 lentivirus aliquots per 2.5×10^5 $CD4^+$ or $CD8^+$ T cells.
6. Add rhIL-2 for a final concentration of 1000 IU/mL for Treg cells, 100 IU/mL for $CD4^+$ T cells, and 300 IU/mL for $CD8^+$ T cells.
7. Centrifuge at 1000 *g* for 1 h at 32 °C.

8. Transfer transduced T cells from 1.5-mL microcentrifuge tubes to a 24-well plate, each 1.5 microcentrifuge tube to one well.
9. Place the plate with transduced T cells in tissue culture incubator.
10. The following day, top up each well of the 24-well to 2 mL RPMI10 complete medium with rhIL-2 (1000 IU/mL for Treg cells, 100 IU/mL for $CD4^+$ T cells, and 300 IU/mL for $CD8^+$ T cells). Continue expanding transduced T cell cultures over time by splitting cells and adding fresh medium and rhIL-2 as needed.

3.4 CAR^+ T Cell Sorting

1. Five days post transduction, count transduced T cells with Trypan Blue.
2. Centrifuge cells at 500 *g* for 5 min at room temperature (RT).
3. Resuspend cells at 15×10^6 cells/mL in DPBS.
4. Filter cell suspension through a 40-μm filter cap into a FACS tube and put on ice.
5. Prepare collection tubes for sorting by adding 3 mL RPMI10 complete medium per 15-mL conical tube and put on ice. Collection tube type and complete medium volume will depend on the specific cell sorting instrument used.
6. Sort CAR reporter-expressing cells, in this case GFP^+, using FACS (Fig. 5).
7. Count the cells post-sort in a 1:1 ratio with Trypan Blue.
8. Plate sorted CAR^+ T cells at 5×10^5 cells per well of a 24-well plate in 1 mL RPMI10 complete medium and rhIL-2 and put in the tissue culture incubator. Continue expanding CAR^+ T cell cultures over time by splitting cells and adding fresh RPMI10 complete medium and rhIL-2 as needed.

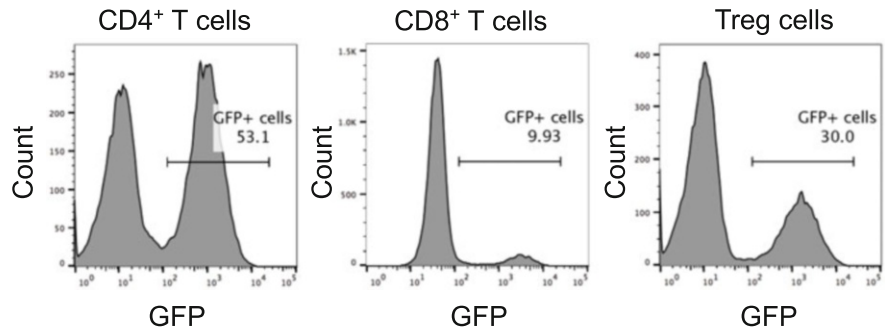

Fig. 5 Purifying human CAR^+ T cells. FACS sorting of CD19CAR-2A-GFP-expressing human $CD4^+$ T cells, $CD8^+$ T cells, and Treg cells one week post transduction

3.5 Immune Assays

Once CAR$^+$ T cells have been generated, the time comes to submit them to a battery of in vitro immune assays to assess CAR functionality. Here, we describe protocols to measure CAR-mediated T cell activation, cytotoxicity, expansion, and exhaustion. In the specific case of CAR$^+$ Treg cells, CAR Treg suppressive function and lineage stability are also assessed.

3.5.1 Activation

T cell activation via the TCR in response to its cognate antigen results in marked changes in T cell size, morphology, and proliferative patterns (Fig. 3). Hence, the first step to demonstrate the functionality of a new CAR is to show that it leads to T cell activation in the presence of its target antigen. To do this, we generate stable K562 cell lines with the CAR target protein on their surface via lentiviral transduction (in this case, CD19-K562), irradiate them to inhibit their proliferation, and co-incubate them with CAR T cells (Fig. 6a). K562 cells are an easy-to-maintain human myelogenous leukemia cell line devoid of MHC and co-stimulatory ligand (CD80 and CD86) expression, providing a blank canvas for CAR target expression. Anti-CD3/CD28 Dynabeads are included as positive control for T cell activation. To quantify activation 48 h post co-incubation, in addition to visual inspection of the CAR$^+$ T cell and CAR target$^+$ cell co-cultures, upregulation of the surface expression of the early activation markers CD25 and CD71 [11] is monitored by flow cytometry (Fig. 6b), and IFN-γ (conventional CD4$^+$ T cells) and IL-10 (Treg cells) levels are measured via enzyme-linked immunosorbent assay (ELISA) in the co-culture supernatants (Fig. 7).

Day 0: Co-culture Plate Setup

1. Collect K562 cell lines (e.g., parental K562 and CD19-K562) into conical tubes.
2. Irradiate K562 cell lines with 4000 rad in cesium-137 irradiator.
3. Collect CAR$^+$ T cells into 15-mL conical tubes and magnetically remove anti-CD3/CD28 Dynabeads by incubating in a DynaMag-15 magnet for 3 min and transferring the debeaded CAR$^+$ T cell suspension into a new 15-mL conical tube.
4. Count irradiated K562 cells and debeaded CAR$^+$ T cells with Trypan Blue.
5. Centrifuge K562 cells and CAR$^+$ T cells at 500 *g* for 5 min at RT.
6. Resuspend cells in RPMI10 complete medium at 10^6 cells/mL.

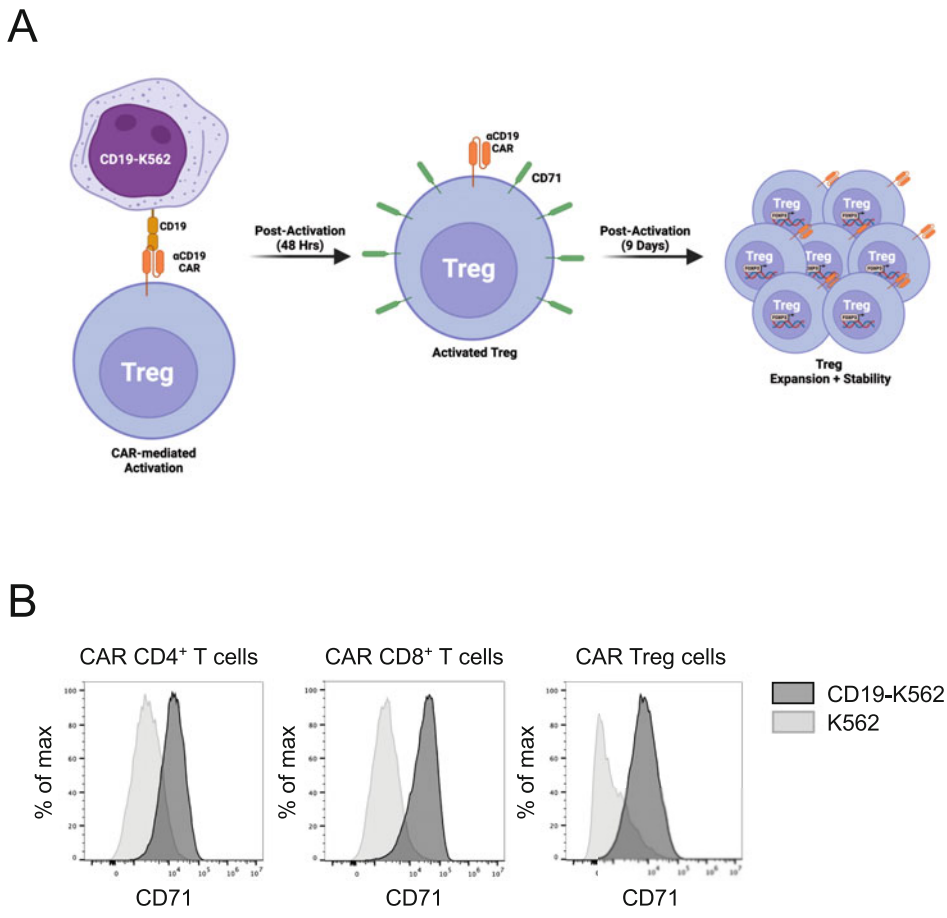

Fig. 6 CAR-mediated activation of human CAR+ T cells. (**a**) The workflow for evaluating CAR-mediated early activation of CAR T cells, e.g., CD19CAR Tregs, involves co-incubating CD19CAR Tregs with irradiated CD19-expressing K562 for 48 h, followed by flow cytometry to detect upregulation of the activation marker CD71 (transferrin receptor). Continued expansion of the cells for 9 days allows for assessment of CAR-mediated expansion (CAR Treg cell number), as well as assess the phenotypic stability of the CAR Tregs (e.g., FOXP3 expression). (**b**) CD19CAR-mediated activation of human $CD4^+$ T cells, $CD8^+$ T cells, and Treg cells after 48 h co-incubation with either irradiated K562 or CD199-K562, as assessed by flow cytometry with anti-human CD71 FITC. Histograms represent CD71 surface expression of live CD4 + GFP+ cells

7. If working with CAR Tregs, add rhIL-2 for a final concentration of 1000 IU/mL. *Do not* add rhIL-2 if working with either CAR $CD4^+$ or CAR $CD8^+$ T cells.
8. Co-incubate 1×10^5 CAR^+ T cells with 1×10^5 parental K562 cells, CAR-antigen-expressing K562 cells, or anti-CD3/CD28 Dynabeads (2.5 μL) in triplicates in a 96-well *round-bottom* plate (1:1 ratio).
9. Incubate plate for 48 h in the tissue culture incubator.

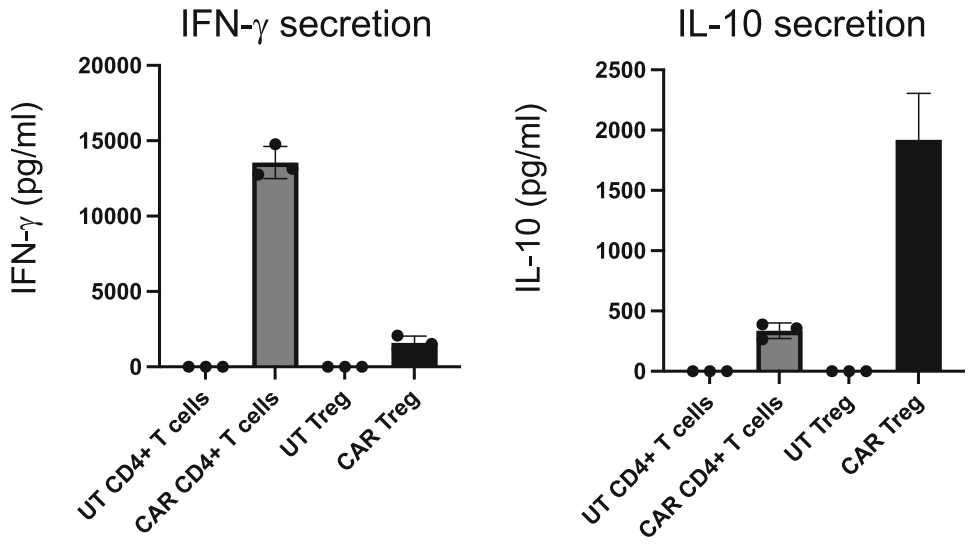

Fig. 7 Cytokine secretion by activated human CAR$^+$ T cells. IFN-γ and IL-10 secretion by either untransduced (UT) or CD19CAR-expressing human CD4$^+$ T cells and Treg cells activated by irradiated CD19-K562 cells for 48 h. Cytokine levels were measured in the co-culture supernatants using enzyme-linked immunosorbent assay (ELISA)

Day 2: Antibody Staining and Flow Cytometry

1. Resuspend and transfer the contents of each 96-well round-bottom plate well into a labeled 1.5-mL microcentrifuge tube.
2. Centrifuge at 500 *g* for 5 min at RT.
3. In the meantime, prepare antibody master mix with DPBS, anti-human CD4 PE/Cy7 1:200, anti-human CD8 PerCP 1:100, anti-human CD25 APC 1:100, anti-human CD71 PE 1:100, and Ghost Viability Dye BV510 1:500. Each sample will require 50 μl of antibody master mix. Hence, if staining 10 samples as an example, add 2.5 μl anti-human CD4 PE/Cy7, 5 μl anti-human CD8 PerCP, 5 μl anti-human CD25 APC, 5 μl anti-human CD71 PE, and 1 μl Ghost Viability Dye BV510 to 0.5 mL DPBS in a 1.5-mL microcentrifuge tube and put on ice in the dark.
4. Carefully collect supernatant from centrifuged microcentrifuge tubes into a new labeled microcentrifuge with a micropipette without disturbing the cell pellet and store at 4 °C if analyzing on the same day or at −20 °C for long-term storage.
5. Briefly vortex cell pellet.
6. Add 50 μL of antibody master mix into each microcentrifuge tube.
7. Briefly vortex and incubate at 4 °C for 30 min in the dark.
8. Wash by adding 500 μL PBS.

9. Centrifuge at 500 *g* for 5 min at RT.
10. Decant supernatant.
11. Resuspend the cell pellet in 300 μL of PBS.
12. Transfer to FACS tubes and put on ice in the dark.
13. Analyze by flow cytometry (*see* **Note 4**). The expected outcome is upregulation of early activation marker surface expression in activated CAR^+ T cells (Fig. 6b).

Day 2: Measure Cytokine Secretion

1. If needed, thaw CAR T cell and K562 cell co-culture supernatants on ice.
2. Follow Human IFN-γ Quantikine ELISA Kit protocol.
3. Measure absorbance in microplate reader. The expected outcome is detection of IFN-γ production by conventional CAR $CD4^+$ T cells but not by CAR Treg cells (Fig. 7).
4. Follow Human IL-10 Quantikine ELISA Kit protocol.
5. Measure absorbance in a microplate reader. The expected outcome is detection of IL-10 production by CAR Treg cells but not by conventional CAR $CD4^+$ T cells (Fig. 7).

3.5.2 Cytotoxicity

The ability to kill antigen-expressing target cells is the most important functional readout for conventional CAR T cells (*see* **Note 5**). In this protocol, we co-incubated CAR^+ T cells with CAR target-expressing K562 in progressively increasing T cell/target cell ratios. CAR T cell cytotoxicity was then determined through the indirect measurement of extracellular lactate dehydrogenase (LDH) activity in the supernatant of co-cultures [12]. LDH is an intracellular enzyme and thus is only present in the extracellular milieu in the event of cell lysis. Addition of a substrate to supernatant samples that is converted into a chromogenic product by LDH allows for accurate quantification of supernatant LDH activity and, by proxy, target cell lysis (Fig. 8).

1. Collect CAR^+ T cells into 15-mL conical tubes and *magnetically remove anti-CD3/CD28 Dynabeads* by incubating in a DynaMag-15 magnet for 3 min and transferring the debeaded CAR^+ T cell suspension into a new 15-ml conical tube.
2. Count CAR^+ T cells and CAR target-expressing K562 cells (e.g., CD19-K562) with Trypan Blue.
3. Obtain 3×10^5 CAR^+ T cells. This allows to cover triplicates of 3 T cell/target cell ratios, 1:1, 1:2, and 1:4, at 5×10^4 target cells per 96-well *round-bottom* plate well, as $5 \times 10^4 x3 + 2.5 \times 10^4 x3 + 1.25 \times 10^4 x3 = 2.63 \times 10^4 \sim 3 \times 10^5$ CAR^+ T cells.
4. Centrifuge at 500 *g* for 5 min at RT.

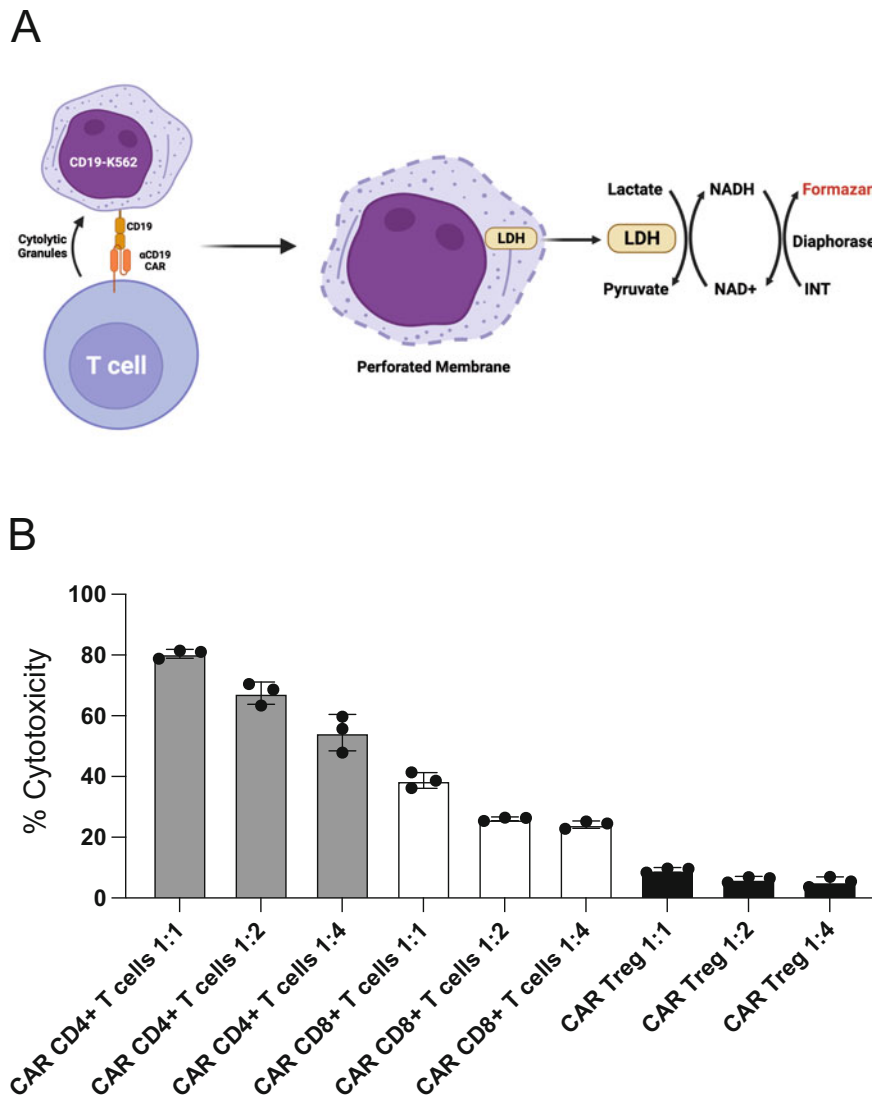

Fig. 8 CAR-mediated cytotoxicity of human CAR$^+$ T cells. (**a**) The workflow for evaluating CAR-mediated cytotoxicity of CAR T cells, e.g., CD19CAR T cells, involves co-incubating CD19CAR T cells with CD19-K562 cells at different ratios. Following co-incubation for a given period of time (e.g., 48 h), the co-culture supernatants are collected and their content in lactate dehydrogenase (LDH, an intracellular enzyme that is only released into the supernatant upon cell lysis) is measured based on a reaction that quantitatively uses LDH to generate a colored product, formazan. (**b**) Relative cytotoxicity of CD19CAR human CD4$^+$ T cells, CD8$^+$ T cells, and Treg cells towards CD19-K562 cells for 48 h co-incubation. The ratios (1:1, 1:2, 1:4) indicate the ratio of CAR T cell number to CD19-K562 cell number per condition

5. Resuspend 3×10^5 CAR$^+$ T cells in 600 μL RPMI10 complete medium. This yields 200 μL for each of the 1:1 triplicates. If working with CAR Tregs, add rhIL-2 to a final concentration of 1000 IU/mL. If working with CAR CD4$^+$ or CD8$^+$ T cells, do not add rhIL-2.

6. Perform serial dilution on the 96-well *round-bottom* plate as follows:
   (a) Transfer 200 μL of CAR T cell suspension into each of the 3 1:1 ratio wells.
   (b) Add 100 μL of RPMI10 complete medium into each of the 1:2 and 1:4 ratio wells.
   (c) For each of the triplicates, transfer 100 μL of cell suspension from the 1:1 wells into the associated 1:2 ratio wells.
   (d) For each of the triplicates, transfer 100 μL of cell suspension, from the 1:2 wells, into the associated 1:4 ratio wells.
   (e) Discard 100 μL of cell suspension from the 1:4 ratio wells into a waste container. Each well now has 100 μL CAR T cell suspension with either 5×10^4 cells (1:1), 2.5×10^4 (1:2), or 1.25×10^4 cells (1:4).
7. Resuspend CAR target-expressing K562 cells at 5×10^5 cells/ml in RPMI10 complete medium. If working with CAR Tregs, add rhIL-2 to a final concentration of 1000 IU/mL. If working with CAR $CD4^+$ or $CD8^+$ T cells, do not add rhIL-2.
8. Add 5×10^5 CAR target-expressing K562 cells in 100 μL on top of every sample well, bringing the volume of each sample well to 200 μL.
9. Add 5×10^5 CAR target-expressing K562 cells in 100 μL to 6 empty wells, and then add 100 μL of RPMI10 complete medium on top. These will be used later as minimum and maximum LDH release controls in triplicate.
10. Incubate for 48 h in the tissue culture incubator.
11. Two days later, add 20 μL of 10× concentrated lysis buffer from the CyQUANT LDH Cytotoxicity Assay kit to three wells with CAR target-expressing K562 cells alone.
12. Incubate for 45 min in the tissue culture incubator.
13. Carefully remove 50 μL of supernatant from each sample using a multichannel pipette into a new 96-well plate.
14. Follow the CyQUANT LDH Cytotoxicity Assay kit protocol.
15. Measure absorbance in a microplate reader. The expected outcome is an increase in supernatant LDH activity, hence target cell lysis, with increased CAR T cell number per well (Fig. 8b). Of note, as this assay utilizes the supernatant and not the co-incubated cells, it allows for sampling multiple time points.

3.5.3 Suppression

Treg cells are a subset of $CD4^+$ T cells dedicated to suppressing immune responses. Treg cells constitute a form of dominant tolerance, directly inhibiting the activation, expansion, and function of effector immune cells. Assessing the capacity of Treg cells to suppress the proliferation of $CD4^+$ and $CD8^+$ T cells in vitro has long

been the method of choice for initial assessment of Treg cell function [13]. In its simplest form, $CD4^+$ and $CD8^+$ T responder (Tresp) cells are labeled with a cell-permeable fluorescent dye, typically carboxyfluorescein succinimidyl ester (CFSE) or, more recently, CellTrace Violet (CTV), and co-incubated with Treg cells at different Treg/Tresp ratios and anti-CD3/28 activating beads (Fig. 9a). Here, we first activate the Tregs via the CAR (with CAR target molecule-expressing irradiated K562 cells) and the Tresp cells via TCR/CD28 (by stimulation with anti-CD3/CD28 Dynabeads) separately overnight (Fig. 9b), as previously reported [14]. The following day, anti-CD3/CD28 Dynabead-activated CTV-labeled Tresp cells are magnetically debeaded, recounted, and added to the CAR-activated Tregs. CTV dilution by Tresp is evaluated 3–5 days later using flow cytometry (Fig. 10).

Day 0: Responder T Cell (Tresp) Fluorescent Dye Staining and Overnight Activation

1. Collect CAR^+ Treg cells, untransduced $CD4^+$ T cells, and untransduced $CD8^+$ T cells into 15-mL conical tubes and magnetically remove anti-CD3/CD28 Dynabeads by incubating in a DynaMag-15 magnet for 3 min and transferring the *debeaded* cell suspensions into new 15-mL conical tubes.
2. Collect K562 cell lines (e.g., parental K562 and CD19-K562) into conical tubes.
3. Irradiate K562 cell lines with 4000 rad in cesium-137 irradiator.
4. Count *debeaded* T cells and irradiated K562 cells with Trypan Blue.
5. Mix untransduced $CD4^+$ T cells and untransduced $CD8^+$ T cells 1:1.
6. Centrifuge mixed T cells at 500 *g* for 5 min at RT.
7. Resuspend T cells in 1 mL DPBS.
8. Add 1 μl CellTrace Violet (CTV) dye reconstituted in DMSO to T cells.
9. Incubate in water bath at 37 °C for 20 min.
10. Add 9 mL of RPMI10 complete medium and centrifuge at 500 *g* for 5 min at RT.
11. Activate CTV-labeled T cells with anti-CD3/CD28 Dynabeads at a *1:10 bead/T cell ratio without rhIL-2*. Strong anti-CD3/CD28 activation and presence of IL-2 greatly decrease the likelihood of the suppression assay working. Assume a 30–50% cell loss with overnight anti-CD3/CD28 T cell overnight activation. Hence, as 2.5×10^4 $CD4^+$ T cells and 2.5×10^4 $CD8^+$ T cells will be needed per well of a full

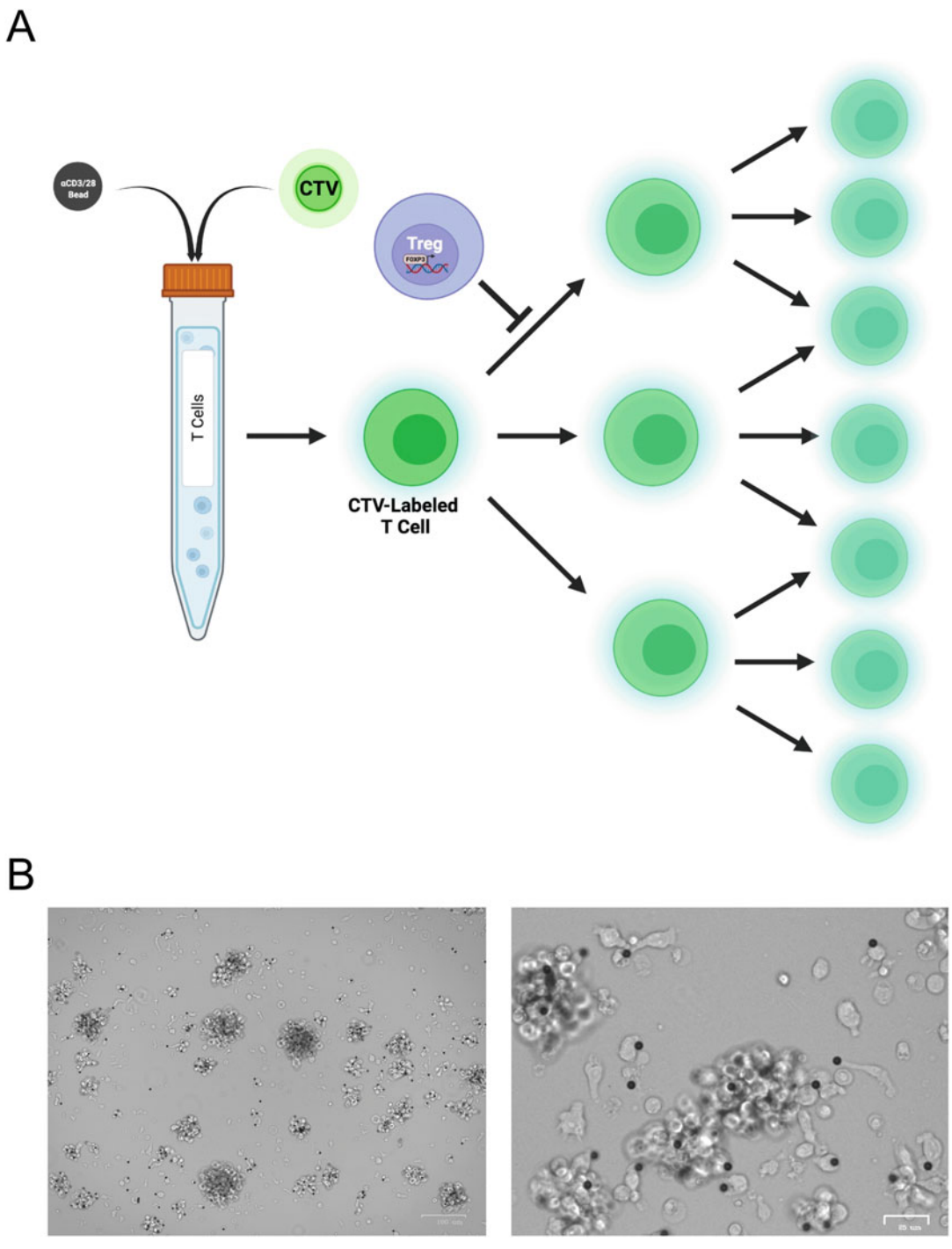

Fig. 9 CAR Treg-mediated suppression assay setup. (**a**) Bulk T cells (human $CD4^+$ T cells and $CD8^+$ T cells mixed at a 1:1 ratio) are labeled with a fluorescent dye, e.g., CellTrace Violet (CTV) and incubated with anti-CD3/CD28 Dynabeads at a 1:10 bead to cell ratio overnight. The following day, these CTV-labeled anti-CD3/CD28-activated T cells are debeaded and co-incubated with CAR-activated CAR Tregs at different Treg/T cell ratio. Suppression of T cell proliferation by CAR Tregs is measured by inhibition of CTV dilution, detected by flow cytometry. (**b**) Anti-CD3/CD28 bead-mediated activation (1:10 bead to cell ratio) of human bulk T cells ($CD4^+$ T cells and $CD8^+$ T cells mixed at a 1:1 ratio) overnight. Images at 5× (left) and 20× (right) magnification

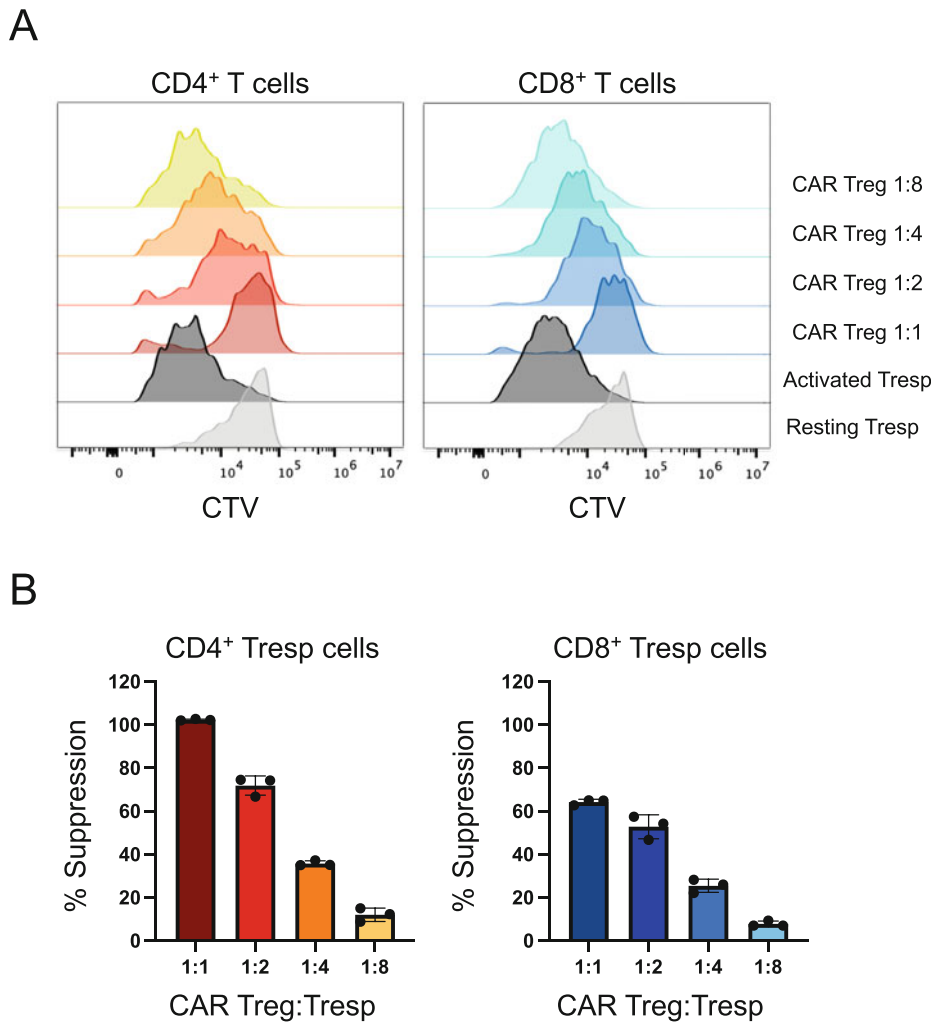

Fig. 10 CAR-mediated suppression of human CAR$^+$ Treg cells. (**a**) CTV dilution by anti-CD3/CD28 bead-activated human T responder (Tresp) cells (human CD4$^+$ T cells and CD8$^+$ T cells mixed at a 1:1 ratio) alone ("activated Tresp") or in the presence of different numbers of CD19CAR Tregs (1:1, 1:2, 1:4), detected using flow cytometry. Unactivated Tresp cells are included as a negative control for proliferation. Data for CD4$^+$ Tresp cells are on the left and data for CD8$^+$ Tresp cells are on the right. CTV dilution for CD4$^+$ Tresp cells is obtained from CD4 + GFP-CTV+ cells (left), whereas CTV dilution for CD4$^+$ Tresp cells is obtained from CD8 + CTV+ cells (right). (**b**) Summary data for histogram plots displayed in (**a**)

96-well *round-bottom* plate, mix 5×10^6 CD4$^+$ T cells with 5×10^6 CD8$^+$ T cells and 10^6 anti-CD3/CD28 Dynabeads (25 μl) in 10 mL RPMI10 complete medium.

12. Incubate in a T25 cell culture flask in the tissue culture incubator overnight.
13. In parallel, add 5×10^4 CTV-labeled *nonactivated* T cells in 200 μl RPMI10 complete medium in three wells of a 96-well *round-bottom* plate as minimum proliferation controls.

Day 1: Co-culture Plate Setup

1. Obtain 9×10^5 CAR^+ Treg cells. This covers triplicates of four CAR Treg/Tresp ratios, 1:1, 1:2, 1:4, 1:8, at 5×10^4 target cells per 96-well *round-bottom* plate well, as 5×10^4x3 + 2.5×10^4x3 + 1.25×10^4x3 = $2.63 \times 10^4 \sim 3 \times 10^5$ CAR^+ Treg cells. The three CAR Treg condition requiring 3×10^5 CAR Tregs each are anti-CD3/CD28 activation (positive control, polyclonal activation), irradiated K562 (negative control, no activation), and irradiated CAR target-expressing K562 (experiment, CAR activation).
2. Obtain 3×10^5 irradiated K562 cells and 3×10^5 irradiated CAR target-expressing K562 cells.
3. Combine 3×10^5 CAR Tregs with 3×10^5 irradiated K562 cells (1:1 ratio), 3×10^5 CAR Tregs with 3×10^5 irradiated CAR target-expressing K562 cells (1:1 ratio), and 3×10^5 CAR Tregs with 6×10^4 anti-CD3/CD28 Dynabeads (1.5 μl, for a 1:10 ratio of beads to Tresp cells, as there will be 12 wells total with 5×10^4 Tresp cells per well, hence 6×10^5 Tresp cells total) in three separate 15-mL conical tubes and centrifuge at 500 *g* for 5 min at RT.
4. Resuspend each one of the cell mixtures above in 600 μL RPMI10 complete medium. This yields 200 μL for each of the 1:1 Treg/Tresp triplicates.
5. Perform serial dilution on the 96-well *round-bottom* plate from earlier as follows:
   (a) Transfer 200 μL of cell suspension into each of the three 1:1 ratio wells.
   (b) Add 100 μL of RPMI10 complete medium into each of the 1:2, 1:4, and 1:8 ratio wells.
   (c) For each of the triplicates, transfer 100 μL of cell suspension from the 1:1 wells into the associated 1:2 ratio wells.
   (d) For each of the triplicates, transfer 100 μL of cell suspension, from the 1:2 wells, into the associated 1:4 ratio wells.
   (e) For each of the triplicates, transfer 100 μL of cell suspension, from the 1:4 wells, into the associated 1:8 ratio wells.
   (f) Discard 100 μL of cell suspension from the 1:8 ratio wells into a waste container. Each well now has 100 μL of cell suspension with either 5×10^4 (1:1), 2.5×10^4 (1:2), 1.25×10^4 (1:4), or 0.625×10^4 (1:8) CAR Tregs and an equal number of either irradiated K562, irradiated CAR target-expressing K562, or 5×10^3 anti-CD3/CD28 Dynabeads.

6. Collect activated $CD4^+$ and $CD8^+$ T cells into a 15-mL conical tube and *magnetically remove anti-CD3/CD28 Dynabeads* by incubating in a DynaMag-15 magnet for 3 min and transferring the *debeaded* T cell suspension into a new 15-mL conical tube.
7. Count *debeaded* T cells with Trypan Blue.
8. Centrifuge 2×10^6 T cells at 500 *g* for 5 min at RT.
9. Resuspend 2×10^6 T cells in 4 mL RPMI10 complete medium.
10. Add 100 μL of T cell suspension (5×10^4 T cells) to each well with CAR Tregs, as well as to three wells with RPMI10 complete medium alone for maximum proliferation controls. The plate now has three wells with nonactivated T cells alone (minimum proliferation control), three wells with activated T cells alone (maximum proliferation control), and activated T cells in the presence of decreasing numbers of CAR Tregs stimulated either via the CAR or via anti-CD3/CD28 Dynabeads.
11. Incubate plate in the tissue culture incubator for 4 days.

Day 5: Antibody Staining and Flow Cytometry

1. Resuspend and transfer the contents of each 96-well round-bottom plate well into a labeled 5-mL FACS tube.
2. Centrifuge at 500 *g* for 5 min at RT.
3. In the meantime, prepare antibody master mix with DPBS, anti-human CD4 PE/Cy7 1:200, and anti-human CD8 PerCP. Each sample will require 50 μl of antibody master mix. Hence, if staining 40 samples as an example, add 10 μl anti-human CD4 PE/Cy7 and 20 μl anti-human CD8 PerCP to 2 mL DPBS in a 15-mL conical tube and put on ice in the dark.
4. Briefly vortex cell pellet.
5. Add 50 μL of antibody master mix into each microcentrifuge tube.
6. Briefly vortex and incubate at 4 °C for 30 min in the dark.
7. Wash by adding 500 μL PBS.
8. Centrifuge at 500 *g* for 5 min at RT.
9. Decant supernatant.
10. Resuspend the cell pellet in 300 μL of PBS.
11. Put tubes on ice in the dark.
12. Analyze by flow cytometry (*see* **Note 6**). The expected outcome is nonactivated $CD4^+$ and $CD8^+$ T cells alone to display a uniformly high CTV fluorescence peak; activated $CD4^+$ and $CD8^+$ T cells alone displaying multiple peaks of CTV intensity, one corresponding to each cell division; and activated $CD4^+$

and $CD8^+$ T cells in the presence of activated CAR Tregs displaying a reduction in the number of CTV peaks, hence in proliferation (Fig. 10a). Treg cell-mediated suppression (Fig. 10b) is calculated as follows:

$$\%\text{suppression} = \frac{\%\text{proliferating Tresp cells alone} - \%\text{proliferating Tresp cells with Tregs}}{\%\text{proliferating Tresp cells alone}}$$

3.5.4 Expansion

A notable indicator of a successful CAR construct is the expansion of the CAR^+ T cell population upon stimulation with the appropriate antigen. Ideally, this expansion will either be comparable to or higher than that induced upon TCR/CD28 stimulation. With an expansive population, more cells will be available for grafting into hosts, which in return may have a more effective response. In this protocol, T cell subsets ($CD4^+$ T cells, $CD8^+$ T cells, and $CD4^+CD25^+CD127^-$ Treg cells) and conditions (untransduced and CAR^+ cells) are activated via either the CAR (with target molecule-expressing irradiated K562 cells) or via TCR/CD28 (with anti-CD3/CD28 Dynabeads). At 9 days post-activation (or day 18 since T cell purification day), each sample receives flow cytometry counting beads and is stained with anti-CD4, anti-CD8, and a viability dye to evaluate expansion and viability via flow cytometry.

Day 0: Co-culture Plate Setup

1. Collect K562 cell lines (e.g., parental K562 and CD19-K562) into conical tubes.
2. Irradiate K562 cell lines with 4000 rad in cesium-137 irradiator.
3. Collect CAR^+ T cells into 15-mL conical tubes and magnetically remove anti-CD3/CD28 Dynabeads by incubating in a DynaMag-15 magnet for 3 min and transferring the debeaded CAR^+ T cell suspension into a new 15-mL conical tube.
4. Count irradiated K562 cells and debeaded CAR^+ T cells with Trypan Blue.
5. Centrifuge K562 cells and CAR^+ T cells at 500 *g* for 5 min at RT.
6. Resuspend cells in RPMI10 complete medium at 10^6 cells/mL.
7. If working with CAR Tregs, add rhIL-2 for a final concentration of 1000 IU/mL. *Do not* add rhIL-2 if working with either CAR $CD4^+$ or CAR $CD8^+$ T cells.

8. Co-incubate 1×10^5 CAR^+ T cells with 1×10^5 parental K562 cells, CAR-antigen-expressing K562 cells, or anti-CD3/CD28 Dynabeads (2.5 μL) in triplicates in a 96-well *round-bottom* plate (1:1 ratio).
9. Incubate plate for 48 h in the tissue culture incubator.

Days 2–9: Co-culture Expansion

1. Two days later, transfer cells from each 96-well round-bottom plate well into a 24-well plate well with 1 mL RPMI10 complete medium. If working with Tregs, include rhIL-2 at 1000 IU/mL. If working with either $CD4^+$ T cells or $CD8^+$ T cells, *do not add rhIL-2*.
2. Continue to expand co-cultures by adding fresh RPMI10 (with rhIL-2, if needed) and splitting into additional 24-well plates as needed.

Day 9: Antibody Staining and Flow Cytometry

1. Resuspend and transfer the contents of each group of 24-well plate wells well into a conical tube.
2. Count cells with Trypan Blue.
3. Transfer up to 10^6 cells to a 5-mL FACS tube.
4. Add 5000 Precision Count Beads (5 μl) per tube.
5. Centrifuge at 500 *g* for 5 min at RT.
6. In the meantime, prepare antibody master mix with DPBS, anti-human CD4 PE/Cy7 1:200, anti-human CD8 PerCP, and Ghost viability dye BV510. Each sample will require 50 μl of antibody master mix. Hence, if staining 10 samples as an example, add 2.5 μl anti-human CD4 PE/Cy7, 5 μl anti-human CD8 PerCP, and 1 μl Ghost viability dye BV510 to 0.5 mL DPBS in a 1.5-mL microcentrifuge tube and put on ice in the dark.
7. Briefly vortex cell pellet.
8. Add 50 μL of antibody master mix to each FACS tube.
9. Briefly vortex and incubate at 4 °C for 30 min in the dark.
10. Wash by adding 500 μL PBS.
11. Centrifuge at 500 *g* for 5 min at RT.
12. Decant supernatant.
13. Resuspend the cell pellet in 300 μL of PBS.
14. Put tubes on ice in the dark.
15. Analyze by flow cytometry (*see* **Note 7**). Precision Count Beads fluoresce in multiple channels and can thus be identified and gated on by their high signal intensity on the diagonal of FITC and PE channels, for example. Since the absolute number of

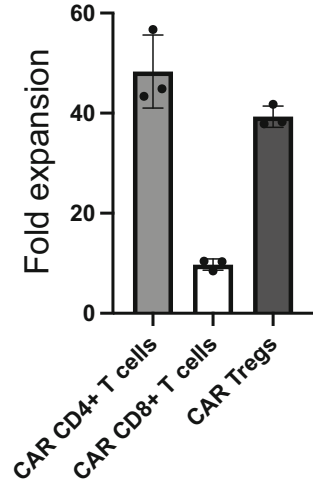

Fig. 11 CAR-mediated expansion of human CAR$^+$ T cells. CAR$^+$ T cell fold-expansion for CD19CAR CD4$^+$ T cells, CD19CAR CD8$^+$ T cells, and CD19CAR Tregs after 9-day co-incubation with irradiated CD19-K562 cells. Absolute cell numbers were calculated using flow cytometry by recording event number for either live CD4$^+$GFP$^+$ or live CD8$^+$GFP$^+$ cells, followed by normalization using precision count beads added number ratio to event number ratio and any dilution factor from using only a fraction of the expanded cells for flow cytometry

beads per tube is known, 5000, these can be used to use the number of events of interest, for instance, live CD4 + GFP+ events, to obtain the absolute number of CAR+ cells. Please ensure to not only normalize the number of cells using the number of Precision Count Beads events per tube, but also to consider the total number of cells per condition vs. the number of cells used for the staining. Different CAR constructs, different blood donors, and different T cell subsets (Fig. 11) are expected to grow at different rates.

3.5.5 *Exhaustion*

Upon constant stimulus from inflammatory conditions or lasting antigen exposure, T cells exhibit exhaustion. T cell exhaustion results in less cytokine production, upregulation of inhibitory signals, growth arrest, metabolic deficiencies, and, eventually, apoptosis. Consequently, exhausted T cells lack the ability to eliminate antigen-expressing cells. When assessing a candidate CAR construct, it is important to evaluate the potential for CAR activation to induce T cell exhaustion. Well-known exhaustion markers include PD-1, CTLA-4, LAG3, TIM-3, and TIGIT [15].

Day 0: Co-culture Plate Setup

1. Collect K562 cell lines (e.g., parental K562 and CD19-K562) into conical tubes.

2. Irradiate K562 cell lines with 4000 rad in cesium-137 irradiator.
3. Collect CAR^+ T cells into 15-mL conical tubes and magnetically remove anti-CD3/CD28 Dynabeads by incubating in a DynaMag-15 magnet for 3 min and transferring the debeaded CAR^+ T cell suspension into a new 15-mL conical tube.
4. Count irradiated K562 cells and debeaded CAR^+ T cells with Trypan Blue.
5. Centrifuge K562 cells and CAR^+ T cells at 500 *g* for 5 min at RT.
6. Resuspend cells in RPMI10 complete medium at 10^6 cells/mL.
7. If working with CAR Tregs, add rhIL-2 for a final concentration of 1000 IU/mL. *Do not* add rhIL-2 if working with either CAR $CD4^+$ or CAR $CD8^+$ T cells.
8. Co-incubate 1×10^5 CAR^+ T cells with 1×10^5 parental K562 cells, CAR-antigen-expressing K562 cells, or anti-CD3/CD28 Dynabeads (2.5 μL) in triplicates in a 96-well *round-bottom* plate (1:1 ratio).
9. Incubate plate for 48 h in the tissue culture incubator.

Days 2–9: Co-culture Expansion

1. Two days later, transfer cells from each 96-well round-bottom plate well into a 24-well plate well with 1 mL RPMI10 complete medium. If working with Tregs, include rhIL-2 at 1000 IU/mL. If working with either $CD4^+$ T cells or $CD8^+$ T cells, *do not add rhIL-2*.
2. Continue to expand co-cultures by adding fresh RPMI10 (with rhIL-2, if needed) and splitting into additional 24-well plates as needed.

Day 9: Antibody Staining and Flow Cytometry

1. Resuspend and transfer the contents of each group of 24-well plate wells well into a conical tube.
2. Count cells with Trypan Blue.
3. Transfer up to 10^6 cells to a 5-mL FACS tube.
4. Centrifuge at 500 *g* for 5 min at RT.
5. In the meantime, prepare antibody master mix with DPBS, anti-human CD4 Alexa 700 1:200, anti-human CD8 APC/Cy7, anti-human PD-1 PerCP, and Ghost viability dye BV510. Each sample will require 50 μl of antibody master mix. Hence, if staining 10 samples as an example, add 2.5 μl anti-human CD4 Alexa 700, 5 μl anti-human CD8 APC/Cy7, 5 μl

anti-human PD-1 PerCP, and 1 μl Ghost viability dye BV510 to 0.5 mL DPBS in a 1.5-ml microcentrifuge tube and put on ice in the dark.

6. Briefly vortex cell pellet.
7. Add 50 μL of antibody master mix to each FACS tube.
8. Briefly vortex and incubate at 4 °C for 30 min in the dark.
9. Wash by adding 500 μL PBS.
10. Centrifuge at 500 *g* for 5 min at RT.
11. Decant supernatant.
12. Resuspend the cell pellet in 300 μL of PBS.
13. Put tubes on ice in the dark.
14. Analyze by flow cytometry (*see* **Note 8**). The expected outcome is the observation of two populations: PD-1-negative and PD-1-positive cells (Fig. 12).

3.5.6 Stability

Treg cells display plasticity, making them susceptible to transdifferentiation into pro-inflammatory effector T cells upon certain environmental cues, including prolonged ex vitro expansion and exposure to inflammatory cytokines [16]. Thus, it is important to ensure that CAR-mediated activation does not compromise Treg stability over time. Established Treg cell functional markers, such as CD25 and CTLA-4, as well as the Treg cell lineage-specific transcription factors FOXP3 and HELIOS, allow for adequate assessment of the phenotypic stability of Tregs over time [16].

Day 0: Co-culture Plate Setup

1. Collect K562 cell lines (e.g., parental K562 and CD19-K562) into conical tubes.
2. Irradiate K562 cell lines with 4000 rad in cesium-137 irradiator.
3. Collect CAR^+ Treg cells into 15-mL conical tubes and magnetically remove anti-CD3/CD28 Dynabeads by incubating in a DynaMag-15 magnet for 3 min and transferring the debeaded CAR^+ Treg suspension into a new 15-mL conical tube.
4. Count irradiated K562 cells and debeaded CAR^+ Treg cells with Trypan Blue.
5. Centrifuge K562 cells and CAR^+ Treg cells at 500 *g* for 5 min at RT.
6. Resuspend cells in RPMI10 complete medium at 10^6 cells/mL.
7. Add rhIL-2 for a final concentration of 1000 IU/mL.

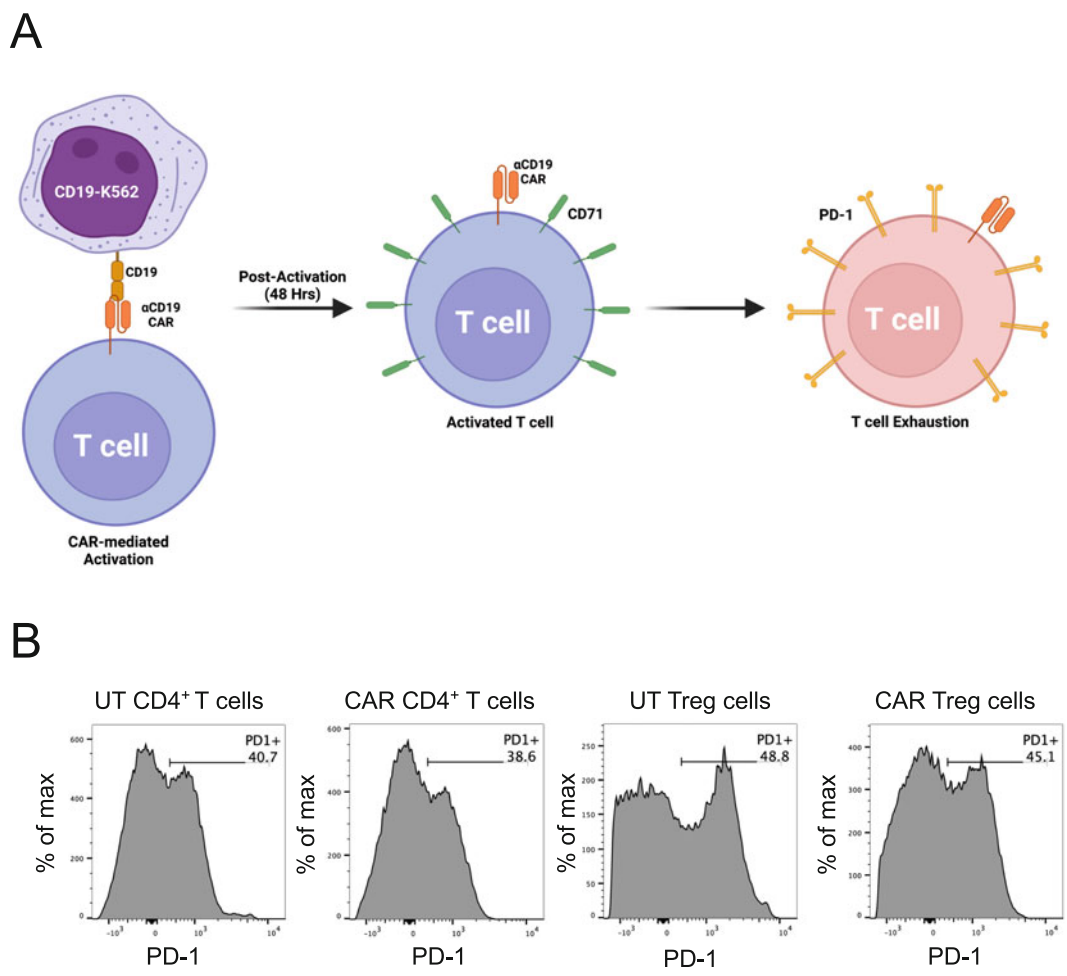

Fig. 12 CAR-mediated exhaustion of human CAR$^+$ T cells. (**a**) The workflow for evaluating exhaustion of CAR T cells, e.g. CD19CAR T cells, involves co-incubating CD19CAR T cells with irradiated CD19-expressing K562 for 9 days, followed by surface staining for PD-1 and detection using flow cytometry. (**b**) Surface PD-1 expression by untransduced (UT) human CD4$^+$ T cells or Treg cells activated via anti-CD3/CD28 beads and by human CD19CAR CD4$^+$ T cells or CD19CAR Treg cells activated either via CD19-K562 cells, as detected by flow cytometry with anti-human PD-1 PerCP. Histograms represent PD-1 expression in live CD4$^+$ cells or CD4$^+$GFP$^+$ cells for UT cells and CD19CAR cells, respectively

8. Co-incubate 1 × 10^5 CAR$^+$ Treg cells with 1 × 10^5 parental K562 cells, CAR-antigen-expressing K562 cells, or anti-CD3/CD28 Dynabeads (2.5 μL) in triplicates in a 96-well *round-bottom* plate (1:1 ratio).
9. Incubate plate for 48 h in the tissue culture incubator.

Days 2–9: Co-culture Expansion

1. Two days later, transfer cells from each 96-well round-bottom plate well into a 24-well plate well with 1 mL RPMI10 complete medium with rhIL-2 at 1000 IU/mL.
2. Continue to expand co-cultures by adding fresh RPMI10 with rhIL-2 at 1000 IU/mL and splitting into additional 24-well plates as needed.

Day 9: Antibody Staining and Flow Cytometry

1. Resuspend and transfer the contents of each group of 24-well plate wells well into a conical tube.
2. Count cells with Trypan Blue.
3. Transfer up to 10^6 cells to a 5 mL FACS tube.
4. Centrifuge at 500 *g* for 5 min at RT.
5. In the meantime, prepare surface staining antibody master mix with DPBS, anti-human CD4 PE/Cy7 1:200, anti-human CD25 APC, and Ghost viability dye BV510. Each sample will require 50 μl of antibody master mix. Hence, if staining 10 samples as an example, add 2.5 μl anti-human CD4 PE/Cy7, 5 μl anti-human CD25 APC, and 1 μl Ghost viability dye BV510 to 0.5 mL DPBS in a 1.5-mL microcentrifuge tube and put on ice in the dark.
6. Briefly vortex cell pellet.
7. Add 50 μL of surface staining antibody master mix to each FACS tube.
8. Briefly vortex and incubate at 4 °C for 30 min in the dark.
9. In the meantime, prepare Fixation/Permeabilization buffer by adding 3 volumes of Fixation/Permeabilization diluent to 1 volume of Fixation/Permeabilization concentrate from the eBioscience Foxp3 transcription factor staining buffer set. Each sample requires 50 μL of Fixation/Permeabilization buffer. Hence, if staining 10 samples, as an example, mix 750 μL of Fixation/Permeabilization diluent with 750 μL of Fixation/Permeabilization concentrate in a 1.5-mL microcentrifuge tube to obtain 1 mL Fixation/Permeabilization buffer and keep on ice.
10. Wash stained cells by adding 500 μL PBS.
11. Centrifuge at 500 *g* for 5 min at RT.
12. Decant supernatant.
13. Briefly vortex cell pellet.
14. Add 50 μL of Fixation/Permeabilization buffer to each FACS tube.
15. Briefly vortex and incubate at 4 °C for 30 min in the dark.

16. In the meantime, prepare Permeabilization buffer by adding 9 volumes of DPBS to 1 volume to Permeabilization buffer 10× concentrate from the eBioscience Foxp3 transcription factor staining buffer set. Each sample requires 500 μL of Permeabilization buffer to wash the Fixation/Permeabilization buffer and 50 μL of Permeabilization buffer for staining with antibodies targeting intracellular proteins. Hence, if staining 10 samples, as an example, mix 9.9 mL DPBS with 1.1 mL of Permeabilization buffer 10× concentrate in a 15-mL conical tube to obtain 11 mL of Permeabilization buffer and keep on ice.
17. Wash fixed and permeabilized cells by adding 500 μL Permeabilization buffer.
18. Centrifuge at 500 *g* for 5 min at RT.
19. In the meantime, prepare intracellular staining antibody master mix with Permeabilization buffer, anti-human FOXP3 eFluor 450 1:40, anti-human HELIOS PE 1:40, and CTLA-4 PerCP-e710 1:40. Each sample will require 50 μl of antibody master mix. Hence, if staining 10 samples as an example, add 12.5 μl anti-human FOXP3 eFluor 450, 12.5 μl anti-human HELIOS PE, and 12.5 μl anti-human CTLA-4 PerCP-e710 to 0.5 mL Permeabilization buffer in a 1.5-mL microcentrifuge tube and put on ice in the dark.
20. Decant supernatant of centrifuged washed cells.
21. Briefly vortex cell pellet.
22. Add 50 μL of intracellular staining antibody master mix to each FACS tube.
23. Briefly vortex and incubate at 4 °C for 30 min in the dark.
24. Wash stained cells by adding 500 μL Permeabilization buffer.
25. Centrifuge at 500 *g* for 5 min at RT.
26. Decant supernatant.
27. Resuspend the cell pellet in 300 μL of PBS.
28. Put tubes on ice in the dark.
29. Analyze by flow cytometry (*see* **Note 9**). The expected outcome is that the majority of CAR Tregs are $FOXP3^{+}HELIOS^{+}$ cells. Use bulk CD4+ T cells as a negative control for FOXP3 and HELIOS staining (Fig. 13).

4 Notes

1. RPMI10 complete medium is prepared by adding 50 mL FBS, 5 mL penicillin–streptomycin solution, 5 mL GlutaMAX, 5 mL sodium pyruvate, 5 mL NEAA, and 5 mL 1 M HEPES to

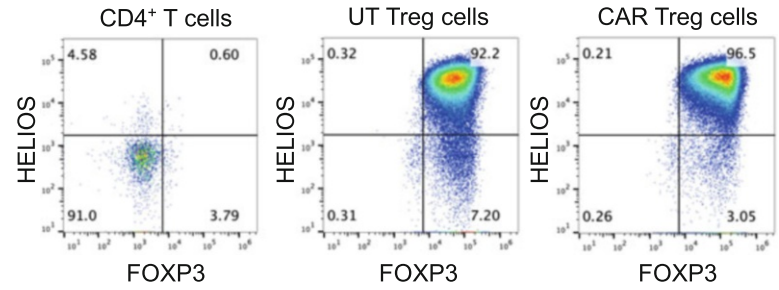

Fig. 13 CAR-mediated activation impact on the stability of human CAR$^+$ Treg cells. Expression of the Treg cell lineage transcription factors FOXP3 and HELIOS in CD4$^+$ T cells activated via anti-CD3/CD28 Dynabeads, untransduced (UT) Treg cells activated via anti-CD3/CD28 Dynabeads, or CD19CAR Treg cells activated with irradiated CD19-K562 cells for 9 days. Dot plots represent FOXP3 and HELIOS expression in viable CD4$^+$ cells, as detected by flow cytometry with anti-human FOXP3 eFluor 450 and anti-human HELIOS PE

500 mL RPMI 1640 medium. After adding all the components to the basal RPMI 1640 medium, RPMI10 can be filtered using a vacuum filtration system with a 20-μm pore size membrane to ensure sterility.

2. While freshly isolated and plated T cells (day 0) have a round morphology, over the course of 48 h following activation (day 2) they acquire an elongated shape, grow in size, and form dense clusters around the anti-CD3/CD28 Dynabeads (Fig. 3).
3. DMEM10 complete medium is prepared by adding 50 mL FBS, 5 mL GlutaMAX, 5 mL sodium pyruvate, and 5 mL NEAA to 500 mL DMEM medium. After adding all the components to the basal DMEM medium, RPMI10 can be filtered using a vacuum filtration system with a 20-μm pore size membrane to ensure sterility. Penicillin–streptomycin and HEPES have both been reported to negatively impact DNA transfection and are thus not included in DMEM10 complete medium.
4. Multicolor flow cytometry requires preparation of single-color controls for the cytometer to perform compensation, i.e., correct for spectral overlap between different channels. Using cells is preferable to using beads for single-color antibody stains, to better match the intensity of the fluorophores used. In this specific case, six single-color controls are needed: unstained, GFP, PE/Cy7, PerCP, BV510, PE, and APC.
5. There are a multitude of in vitro cytotoxicity assays with readouts based on radioactivity, microscopy, flow cytometry, luminescence, and absorbance. The most cost-effective and scalable readout is absorbance, which we used in this protocol.

6. In this specific case, five single-color controls are needed for compensation: unstained, GFP, PE/Cy7, PerCP, and CTV.
7. In this specific case, five single-color controls are needed for compensation: unstained, GFP, PE/Cy7, PerCP, and BV510.
8. In this specific case, six single-color controls are needed for compensation: unstained, GFP, Alexa 700, APC/Cy7, PerCP, and BV510.
9. In this specific case, eight single-color controls are needed for compensation: unstained, GFP, PE/Cy7, APC, BV510, eFluor 450, PE, and PerCP-e710.

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Chapter 16

How to Test Human CAR T Cells in Solid Tumors, the Next Frontier of CAR T Cell Therapy

Russell W. Cochrane, Andrew Fiorentino, Eva Allen, Rob A. Robino, Jaime Quiroga, and Leonardo M. R. Ferreira

Abstract

Chimeric antigen receptor (CAR) T cell therapy has proven to be a successful treatment option for leukemias and lymphomas. These encouraging outcomes underscore the potential of adoptive cell therapy for other oncology applications, namely, solid tumors. However, CAR T cells are yet to succeed in treating solid tumors. Unlike liquid tumors, solid tumors create a hostile tumor microenvironment (TME). CAR T cells must traffic to the TME, survive, and retain their function to eradicate the tumor. Nevertheless, there is no universal preclinical model to systematically test candidate CARs and CAR targets for their capacity to infiltrate and eliminate human solid tumors in vivo. Here, we provide a detailed protocol to evaluate human CAR $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in immunodeficient (NSG) mice bearing antigen-expressing human solid tumors.

Key words T cell, Chimeric antigen receptor, Synthetic immunology, Cancer, Solid tumor, Model, Microenvironment, Trafficking, Infiltration

1 Introduction

Chimeric antigen receptors (CARs) are innovative synthetic immune receptors that have revolutionized the adoptive cell therapy field. CARs comprise three components: antigen binding extracellular domain, intracellular signaling domain, and transmembrane domain. Each domain is customizable for a given cell type, target, and function [1]. Extensive research has been conducted using CARs to redirect T cells to antigens on the surface of cancer cells [2–4]. Typically, T cells are restricted to seeing antigens presented by the target cell's major histocompatibility complex (MHC) [5]. Researchers have leveraged T cells by providing them with a new specificity determined by a CAR to eliminate elusive cancer cells that typically hide from the immune system. Additionally, the customization of signaling domains of the

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CAR constructs has allowed researchers to alter or improve T cell function.

Recently, CAR T cell therapy was approved for treating leukemias and lymphomas with revolutionizing results [6]. However, the translation of CAR technology for treating solid tumors has not reached the same level of success [7]. Unlike in the treatment of blood cancers, CAR T cells must traffic successfully to the solid tumor site, the so-called tumor microenvironment (TME). They must also penetrate the stromal components of the solid tumor and evoke a specific cytotoxic response against tumor antigens. However, solid tumors may selectively lose expression of chemokines, vascular-related factors, and other molecules that would otherwise allow for the recruitment and retention of circulating T cells. Moreover, solid tumor-associated antigens are either expressed at lower levels, less predominant, and/or not as unique as those found in liquid tumors. Even after success in all these aspects, CAR T cells must now survive the harsh environment of the TME, characterized by nutrient scarcity, hypoxia, and low pH [8, 9]. Luckily, the customizable nature of the CAR platform provides researchers the opportunity to develop a construct that may fulfill all these requirements for successful treatment.

Once a researcher has developed a CAR construct, it is of utmost importance to test the resulting CAR T cells' ability to traffic to and kill solid tumors in vivo. The CAR candidate may have worked in vitro, but this does not necessarily mean it will work in vivo [10, 11]. It may not possess the required affinity, expression levels, and/or signaling strength required for CAR T cells to traffic to the site of tumor growth, survive in a complex environment where conditions may be more representative of a human tumor, and eliminate a three-dimensional tumor. Therefore, the ability of CAR T cells to traffic to a solid tumor ought to be tackled before addressing any further issues pertaining to the TME's immunosuppressive factors. However, there is no universal model to evaluate human CAR T cell trafficking to a solid human tumor in vivo, with most studies solely measuring the effect of infused CAR T cells on tumor growth without investigating the infiltration of CAR T cells in the tumors.

Here, we provide a detailed protocol to test CAR constructs in human $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells in immunodeficient (NSG) mice bearing solid human tumors. This model provides information not only on the trafficking capacity of CAR T cells but also on their ability to survive in the metabolically harsh conditions of a TME and to reduce tumor burden.

2 Materials

2.1 T Cell Isolation

- 1/10 leukopak (STEMCELL Technologies #200-0092).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- RPMI 1640 medium, no glutamine (Gibco #11875093).
- Fetal bovine serum (FBS) (Gibco #26140079).
- Penicillin–streptomycin solution (Gibco #15140122).
- GlutaMAX (Gibco #35050061).
- Sodium pyruvate (Gibco #11360070).
- Nonessential amino acid (NEAA) solution (Gibco #11140050).
- 1 M HEPES (Gibco #15630080).
- Ammonium chloride solution (STEMCELL Technologies #07850).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- 0.5 M EDTA, pH 8.0 (Gibco #15575020).
- EasySep magnet (STEMCELL Technologies #18000).
- EasySep Human $CD8^+$ T Cell Enrichment Kit (STEMCELL Technologies #19053).
- EasySep Human $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies #19052).
- Easy 50 EasySep magnet (STEMCELL Technologies #18002).
- Tissue culture 24-well plates (VWR #10861–558).
- Human CD3/28 T Cell Expansion and Activation Dynabeads (Gibco #11131D).
- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200–02).

2.2 Transduction

- T cells isolated and activated in Subheading 2.1.
- Titrated CAR-encoding lentivirus.
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- RPMI10 complete medium (*see* Subheading 2.1).

- Recombinant human interleukin-2 (rhIL-2) (Peprotech #200-02).
- Flow cytometer (e.g., Beckman CytoFLEX).

2.3 K562 Subcutaneous Injection

- 8–12-week NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ (NSG) mice (the Jackson Laboratory Strain #005557).
- 70% ethanol (VWR #97064-768).
- Hair clipper (Wahl #79608).
- Alcohol swabs (BD #326895).
- Scale (Mettler-Toledo #PB602-S).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Digital caliper (Fisher Scientific #15-077-957).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Insulin syringes 3/10 mL 8 mm 31G (BD #328438).

2.4 CAR T Cell Intravenous Injection

- Digital caliper (Fisher Scientific #15-077-957).
- Mouse ear tags (World Precision Instruments #501893).
- Easy 50 EasySep magnet (STEMCELL Technologies #18002).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Insulin syringes 3/10 mL 8 mm 31G (BD #328438).
- Isoflurane 99.9% (McKesson #803250).
- Low-Flow Anesthesia System for Mice and Rats (Kent Scientific SomnoSuite, Fisher Scientific #13-005-111).
- Heating pad (Kent Scientific DCT-25).

2.5 Solid Tumor Measurement

- 70% ethanol (VWR #97064-768)
- Digital caliper (Fisher Scientific #15-077-957).

2.6 Tissue Dissection

- Digital caliper (Fisher Scientific #15-077-957).
- 70% ethanol (VWR #97064-768).
- Scale (Mettler-Toledo #PB602-S).
- Hair clipper (Wahl #79608).

- Forceps (VWR #82027-406).
- Surgical scissors (VWR #470315-214).
- Scalpel (VWR #82029-850).
- 100 × 15 mm Petri dish (VWR #25384-070).
- 60 × 15mm Petri dish (VWR #25384-164).
- RPMI 1640 medium, no glutamine (Gibco #11875093).

2.7 Tissue Processing

- 100 × 15 mm Petri dish (VWR #25384-070).
- 60 × 15 mm Petri dish (VWR #25384-164).
- Heated shaker (VWR # 76407-112).
- Scale (Mettler-Toledo #PB602-S).
- Razor blades (VWR #55411-050).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- 70-μm cell filters (VWR #76327-100).
- 100-μm cell Filters (VWR #76327-102).
- RPMI 1640 medium, no glutamine (Gibco #11875093).
- RPMI10 complete medium (*see* Subheading 2.1).
- Collagenase P from *Clostridium histolyticum* (Roche #11215809103).
- DNase I, RNase Free (NEB M0303L).
- EDTA (Invitrogen #15575–038).
- 5-mL disposable Luer-Lok syringes (Thermo #S7510-5).
- ACK lysing buffer (Gibco #A1049201).
- Trypan Blue solution (Sigma #T8154-100ML).
- Cell counter (TC20 Automated Cell Counter, Bio-Rad #1450102).
- Cell Counting Slides (Bio-Rad #1450016).

2.8 Flow Cytometry

- 5-mL FACS tubes (VWR #76449-666).
- Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) (Gibco #14190144).
- Anti-human CD4 Alexa Fluor 700 (clone SK3, Biolegend #344621).
- Anti-human CD8 PerCP (clone SK1, Biolegend #344707).
- Anti-human CD3 PE Cy7 (clone SK7, Biolegend #344815).
- Ghost Dye Brilliant Violet 510 (TONBO #13-0870-T100).
- 5-mL FACS tubes with 35-μm filter caps (VWR #76449-658).
- Flow cytometer (e.g., Beckman CytoFLEX).

3 Methods

The present protocol utilizes primary human T cells isolated from peripheral blood to generate CAR T cells CAR antigen-expressing K562 cells, an HLA null human myelogenous leukemia cell line, as target cells for the CAR T cells, and NSG mice to test human CAR T cells in a CAR antigen-expressing K562 solid tumor in vivo (Fig. 1a). This requires careful coordination to time the (1) generation, purification, and testing of antigen-expressing K562 cells, (2) injection of antigen-expressing K562 cells into immunodeficient NSG mice and await solid tumor formation, and (3) T cell purification from human peripheral blood, e.g., in the form of a concentrated leukocyte product – leukopak, activation, and transduction with CAR genes (Figs. 1b and 2).

3.1 T Cell Isolation

3.1.1 Leukopak Processing

1. Dilute leukopak in an equivalent volume of DPBS +2% FBS. Mix by pipetting slowly.
2. Centrifuge at 300 *g* for 10 min at room temperature (RT) with the brake *on*.
3. Aspirate supernatant without disturbing the cell pellet.
4. Resuspend the cell pellet, add 2 mL DPBS +2% FBS, and pipette up and down.
5. Add 8 mL ammonium chloride solution to cell suspension (4:1 ratio). Mix by pipetting slowly.
6. Incubate on ice for 15 min.
7. Centrifuge at 500 *g* for 10 min at RT with the brake *on*.
8. Aspirate supernatant.
9. Wash by adding 30 mL DPBS +2% FBS.
10. Centrifuge at 150 *g* for 10 min at RT with the brake *off*.
11. Aspirate supernatant and resuspend the cell pellet in 30 mL DPBS +2% FBS.
12. Count cells by diluting them first 1:100 with DPBS (10 μl cell suspension +90 μl DPBS, and then 10 μl cells diluted 1:10 + 90 μl DPBS) and then 1:1 with Trypan Blue solution (10 μl cells diluted 1:100 + 10 μl Trypan Blue). *See* **Note 1**.

3.1.2 $CD4^+$ T Cell Enrichment (Negative Selection)

1. Collect 15×10^6 PBMCs (peripheral blood mononuclear cells) and centrifuge at 500 *g* for 5 min at RT. The starting cell number will depend on your specific needs.
2. Resuspend cells in Cell Separation Buffer (DPBS +10 mM EDTA +2% FBS) at 50×10^6 cells/mL.
3. Follow EasySep Human $CD4^+$ T cell Enrichment Kit protocol.
4. Count cells in a 1:1 ratio with Trypan Blue.

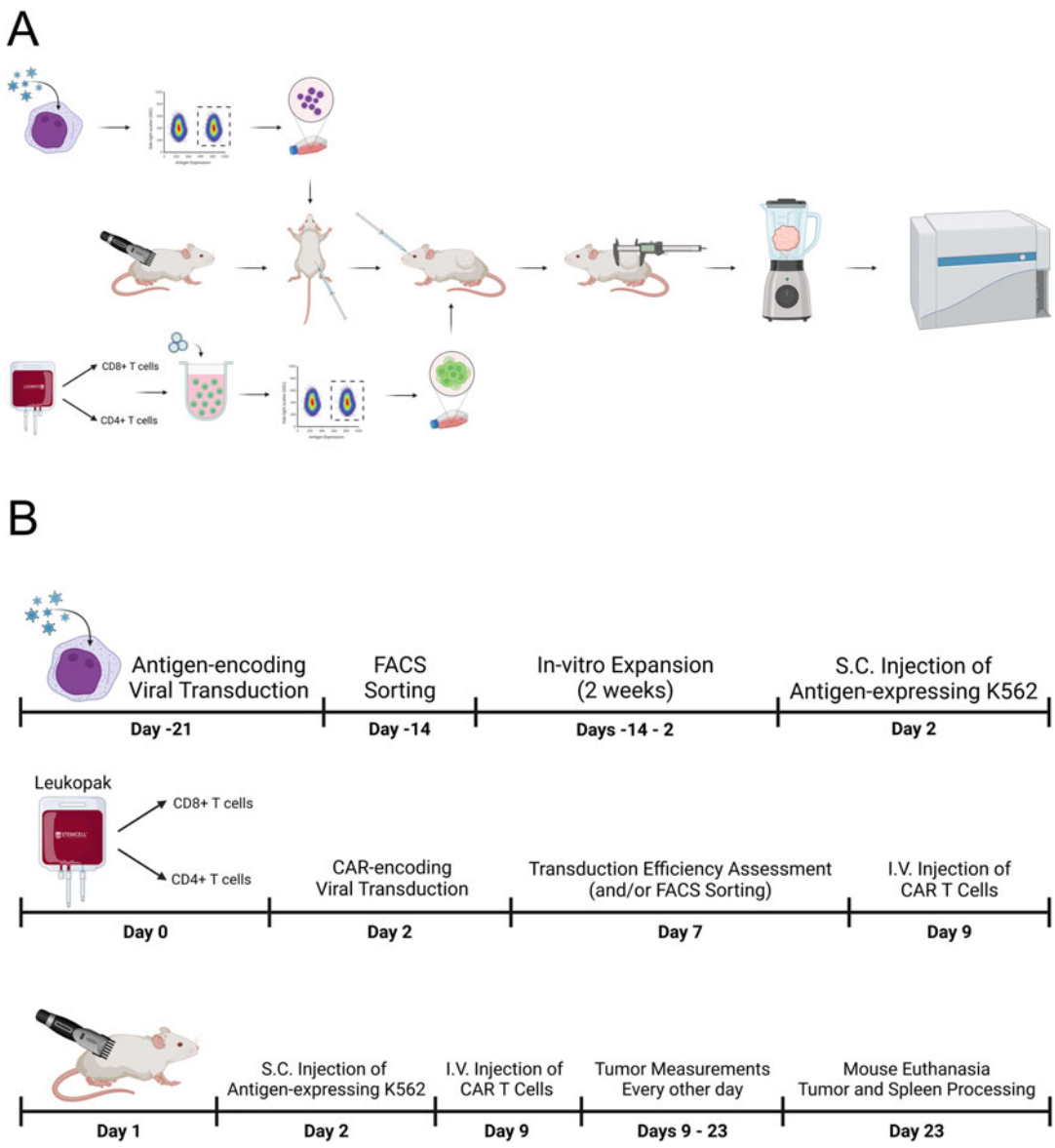

Fig. 1 Experimental timeline. (**a**) Steps needed to evaluate candidate CAR construct efficiency in human solid tumors. These can be divided into three components: generation of CAR target-expressing tumor cells, production of human CAR T cells, and NSG mouse experimentation. (**b**) Experimental timeline separating the three components of the experiment. Denoted days are based on the isolation of primary human T cells, the most time-sensitive component. Day 0 indicates leukopak arrival and human T cell isolation. The experimental timeline assumes the antigen-encoding tumor cell line has yet to be produced. Hence, a pure population of antigen-encoding tumor cells should be obtained 3 weeks before leukopak arrival to allow enough time to obtain the required number of tumor cells to inject on day 2

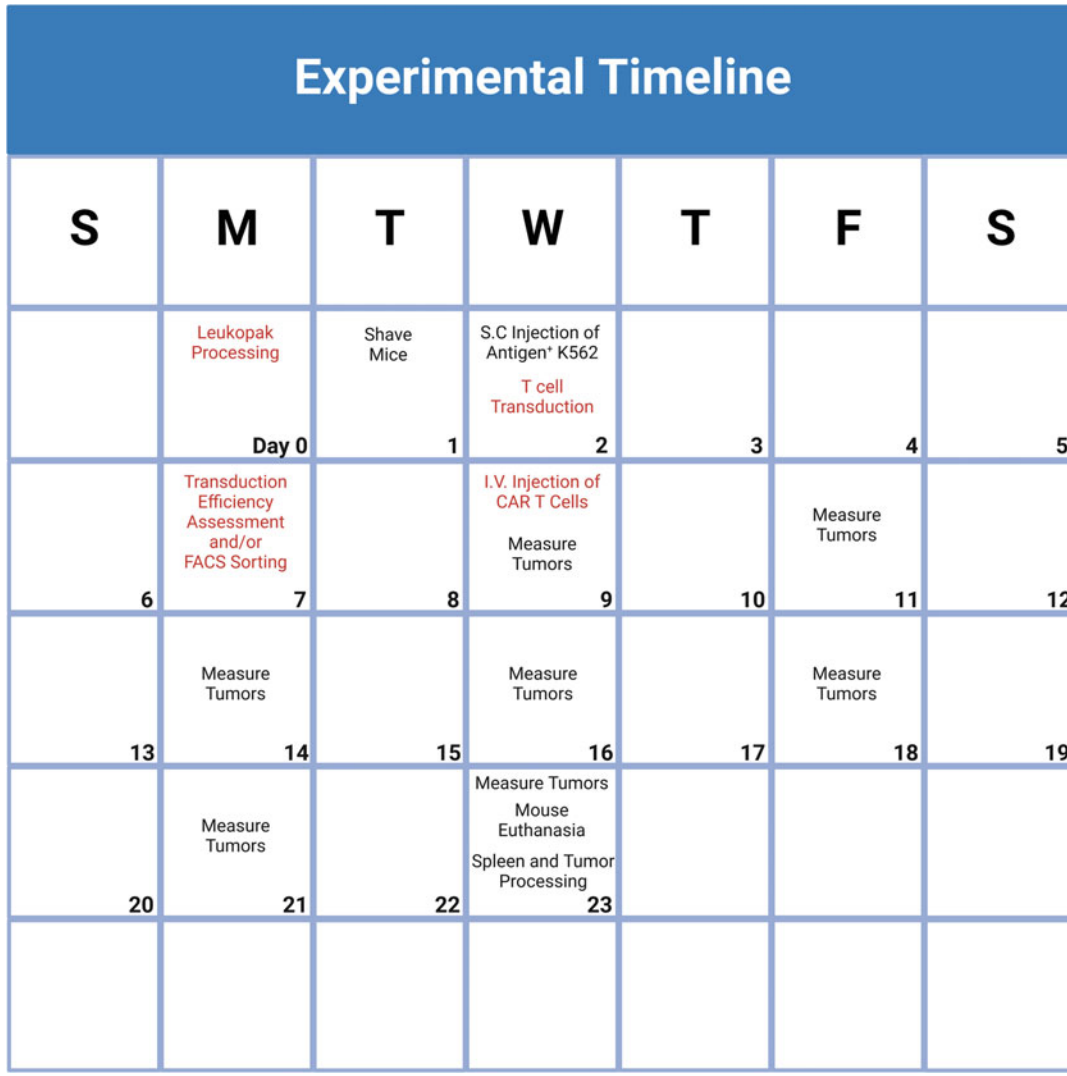

Fig. 2 Calendar view of experimental timeline. (**a**) Experimental timeline in a calendar format to provide a representative experiment weekly schedule. Antigen$^+$ K562 cells are assumed to be already in place. Red text indicates T cell processing, while black text indicates mouse work

3.1.3 CD8$^+$ T Cell Enrichment (Negative Selection)

1. Collect 50 × 10^6 PBMCs and centrifuge at 500 *g* for 5 min at RT. The starting cell number will depend on your specific needs.
2. Resuspend cells in Cell Separation Buffer (DPBS +10 mM EDTA +2% FBS) at 50 × 10^6 cells/ml.
3. Follow EasySep Human CD8$^+$ T cell Enrichment Kit protocol.
4. Count cells in a 1:1 ratio with Trypan Blue.

3.1.4 T Cell Activation and Culture

1. After T cell subset purification and counting, activate $CD4^+$ and $CD8^+$ T cells separately in 24-well plates with Human T-Activator CD3/28 for T Cell Expansion and Activation Dynabeads and recombinant human IL-2 (rhIL-2) in RPMI10 complete medium by conducting the following steps:
2. Centrifuge T cells at 500 *g* for 5 min at RT.
3. Decant supernatant.
4. Resuspend at 5×10^5 T cells per mL of RPM10 complete medium in a conical tube.
5. Add 25 μL anti-CD3/CD28 Dynabeads for every 10^6 T cells for a final 1:1 ratio of Dynabeads to T cells.
6. Add recombinant human IL-2:
   (a) 100 IU/mL of rhIL-2 to $CD4^+$ T cells
   (b) 300 IU/mL of rhIL-2 to $CD8^+$ T cells.
7. Pipette up and down 2–3 times to evenly resuspend T cells, Dynabeads, and rhIL2.
8. Plate 1 mL per well of a 24-well plate so that a total of 5 × 106 T cells are plated per well.
9. Incubate for 48 h in a 37 °C 5% CO_2 tissue culture incubator. *See* **Note 2**.

3.2 Transductions

3.2.1 T Cell Transduction

Once T cells have been activated for 48 h, they are at the peak of activation, ready to be transduced with the CAR construct of interest (*see* **Note 3**). For increased transduction efficiency it is important to transduce the T cells during this time window. There is no need to remove the Dynabeads for this process.

1. Count T cells with Trypan Blue.
2. Centrifuge at 500 *g* for 5 min at room temperature (RT).
3. Resuspend in RPMI10 complete medium at 1.25×10^6 cells/mL.
4. Thaw lentivirus aliquots on ice.
5. Add lentivirus at a multiplicity of infection (MOI) of 2 to 2.5×10^5 T cells in 200 μl in a 1.5-mL microcentrifuge tube. *See* **Note 4**.
6. Add rhIL-2 for a final concentration of 100 IU/mL for $CD4^+$ T cells and 300 IU/ml for $CD8^+$ T cells.
7. Centrifuge at 1000 *g* for 1 h at 32 °C.
8. Transfer transduced T cells from 1.5-mL microcentrifuge tubes to a 24-well plate, each 1.5-microcentrifuge tube to one well.
9. Place the plate with transduced T cells in tissue culture incubator overnight.

10. The following day, bring volume in each well up to 2 mL with RPMI10 complete medium with rhIL-2.
11. Expand and maintain T cells at 5×10^5 to 1×10^6 cells per ml for 1 week. Additionally, provide rhIL-2 every 48 h for a final concentration of 100 IU/mL for $CD4^+$ T cells and 300 IU/mL for $CD8^+$ T cells.
12. On day 7 of T cell culture (Fig. 1b), if a pure CAR^+ population is required, sort CAR^+ cells using fluorescence assisted cell sorting (FACS) to purify cells expressing a CAR reporter gene, e.g., GFP. If a pure CAR T cell population is not possible or needed, calculate the CAR^+ T cell percentage in cultures using flow cytometry.

3.2.2 K562 Culture and Transduction

Due to the large amount of target-expressing K562 required for this experiment (5×10^6 cells per mouse), it is essential to produce and expand the tumor cells well before leukopak arrival. It is crucial to have enough target-expressing K562 on day 2 of T cell culture (Fig. 1b) to minimize the consequences of using older primary T cells. Thus, it is recommended to start expanding target-expressing K562 cells 14 days prior to leukopak arrival. Of note, the selected level of antigen expression on the K562 stable cell line can have an impact: too high expression may not be representative of a clinical phenotype, while too low expression might fail to trigger CAR T cell activation.

1. Three weeks (21 days) before leukopak arrival, count parental K562 cells with Trypan Blue and acquire 2×10^5 cells.
2. Centrifuge at 500 *g* for 5 min at RT.
3. Aspirate supernatant.
4. Resuspend cell pellet with lentivirus aliquot in a 1.5-mL Eppendorf tube.
5. Centrifuge at 1000 *g* for 1 h at 32 °C.
6. Transfer cells to a well of a 24-well plate and put in tissue culture incubator.
7. Expand transduced K562 cells in a T75 flask in preparation for FACS.

3.2.3 Antigen$^+$ K562 Sorting

1. One week (7 days) post transduction, count transduced K562 cells with Trypan Blue.
2. Centrifuge at 500 *g* for 5 min at RT in conical tube.
3. Aspirate supernatant.
4. Surface stain for the antigen of interest using 1 μL antibody/10^6 cells in 100 μl DPBS.
5. Incubate at 4 °C for 30 min protected from light.

6. Wash by adding 500 μL PBS.
7. Centrifuge at 500 *g* for 5 min at RT.
8. Aspirate supernatant.
9. Resuspend pellet in DPBS at 15×10^6 cells/mL for sorting.
10. Filter cell suspension through a 40-μm filter cap into a FACS tube and put on ice.
11. Prepare collection tubes for sorting by adding 3 mL RPMI10 complete medium per 15-mL conical tube and put on ice.
12. Sort antigen$^+$ K562 cells using FACS.
13. Count sorted cells post-sort with Trypan Blue.
14. Plate sorted antigen$^+$ K562 cells at 5×10^5 cells per well of a 24-well plate in 1 mL RPMI10 complete medium and put in the tissue culture incubator.
15. Expand antigen$^+$ K562 cells in a T75 cell culture flask for injection, liquid nitrogen stock freezing, and irradiation. *See* **Note 5**.

3.3 K562 Subcutaneous Injection

Two days after T cell isolation, target positive-K562 cells are subcutaneously injected into the right flank of NSG mice (Figs. 1 and 2). Tumors are allowed to grow to a volume of 50 mm^3 to 150 mm^3 until CAR T cell injection. This will take approximately 7 days.

1. Six days before CAR T cell injections, measure tumor size with a caliper. This is done by measuring tumor length (along the animal, longitudinally) and tumor width (sideways, transversally).
2. Calculate tumor sizes using the formula: tumor volume $= 0.5 \times (\text{length} \times \text{width}^2)$.
3. Randomize NSG mice to be used for each condition based on tumor size.
4. Hold each mouse with one hand sanitize the shave area with 70% ethanol prep.
5. Shave the right flank of each mouse with hair clipper.
6. Ear tag each mouse.
7. Weigh each mouse for an initial weight measurement.
8. Collect 5×10^6 antigen$^+$ K562 cells per mouse being injected. *See* **Notes 6** and **7**.
9. Wash cells by adding two times the volume of DPBS.
10. Centrifuge cells at 500 *g* for 5 min at RT.
11. Decant supernatant.
12. Resuspend cells in ice-cold DPBS at 5×10^7 cells/mL.

13. Aliquot 100 μl of cells into 1.5-mL microcentrifuge tubes so that each tube contains 5×10^6 cells/mL. This will help reduce the variability of tumor growth across mice.
14. Store cells on ice.
15. Relocate to the clean mouse facility room where NSG mice are held, keeping cells on ice.
16. Briefly vortex each tube with K562 cells immediately before injection.
17. Fill a fresh 0.3-mL syringe with attached 31G × 5/16″ mm needle with cells from one aliquot. Do not allow syringe loaded with cell suspension to sit longer than a few minutes. Before injection, gently flick syringe to ensure cells are in suspension and there is no air.
18. Using thumb and index finger, scruff mouse such that flanked skin is taut.
19. With the other hand, fully slide needle at a 10-degree angle with bevel up underneath the skin near the right thigh of the mouse.
20. Gently pull up on the needle to form a "skin tent" to ensure injection is in the subcutaneous space.
21. In one continuous motion, slowly inject 100 μl cell suspension. A "bulge" should be visible where the cell suspension is located. It is important for "bulge" to be far from the injection site as mice tend to rub the area after injection and cause leakage.
22. Slowly withdraw the needle, not allowing leakage of any cells.
23. Place mouse back into cage.
24. Repeat **steps 16–23** for each mouse.
25. Follow up 7 days later, on the day of CAR T cell injection, to measure tumor volumes and identify those between 50 mm^3 and 150 mm^3.

3.4 CAR T Cell Intravenous Injection

One week (7 days) after tumor injections, either saline (DPBS) or 1×10^6 CAR^+CD4^+ and 1×10^6 CAR^+CD8^+ T cells are retro-orbitally intravenously injected into randomized mice bearing 50–150 mM^3 tumors. Tumor volumes are then measured with a caliper every other day. Two weeks (14 days) after CAR T cell injection, i.e., 3 weeks (21 days) after tumor injection, mice are euthanized and tumors surgically removed, weighed, digested, and analyzed via flow cytometry.

1. Collect T cells. Ensure cells are in the logarithmic growth phase by harvesting cells from flasks between 50 and 80% confluent and at least 70% viable.

2. Remove Dynabeads with EasySep magnet for 5 min.
3. Count cells with 1:1 Trypan Blue.
4. Wash by adding two times volume of DPBS.
5. Centrifuge at 500 *g* for 5 min at 32 °C.
6. Resuspend cells at 20×10^6 CAR^+ cells (either $CD4^+$ T cells or $CD8^+$ T cells) per ml of DPBS. If you did not FACS sort CAR^+ cells, make sure to calculate CAR^+ cell number by using transduction efficiency values and total cell numbers.
7. For each mouse being injected with CAR T cells, make a separate 1.5-mL tube with 50 μl with 1×10^6 CAR^+ $CD4^+$ T cells and 50 μl with 1×10^6 CAR^+ $CD8^+$ T cells.
8. Store on ice.
9. Relocate to mouse facility clean room where NSG mice are held while keeping cells on ice throughout process.
10. Anesthetize mice by gas anesthesia (3% isoflurane) in a Plexiglas apparatus.
11. Briefly vortex CAR^+ T cells.
12. Load a fresh 0.3-mL syringe with attached 31G × 5/16′ mm needle with an aliquot of CAR T cells or saline (DPBS). Injected volumes should not exceed 150 μl or contain air bubbles. Do not allow syringes with cells to sit longer than a few minutes. Before injection, gently flick syringe to ensure cells are in suspension.
13. Once mouse is properly anesthetized, as determined by breathing pattern and paw pinching, remove the mouse from Plexiglass apparatus and place it on a nose cone providing 1.8% isoflurane.
14. To prevent hypothermia, place mouse on a warming pad at 37 °C.
15. With thumb and index finger, protrude the anesthetized mouse's eyeball by applying gentle downward pressure.
16. With the needle bevel facing down, not towards eyeball, insert the needle behind the eyeball at a 45-degree angle to the nose.
17. Without poking the eyeball, follow the edge of the eyeball until the needle tip reaches the back end of the eyeball.
18. In one continuous slow motion, inject the cells into the retrobulbar sinus.
19. After a moment, very gently remove needle,
20. Close the eyelid and apply gentle pressure.
21. Place mouse in an individual lying on its back.
22. Monitor mouse until it recovers from anesthesia, should take ca. 60 s.

23. Repeat **steps 10–22** for each mouse.
24. Follow up in 48 h to begin measuring tumor volumes every other day.

3.5 Solid Tumor Measurements

After CAR T cells have been injected, it is important to monitor tumor volume growth over time. It is recommended to measure the tumor volumes every other day after CAR T injection. CAR T cells are not expected to influence tumor volume until 5–7 days after intravenous injection, yet still measure before then to record natural variability in tumor growth (Fig. 3).

1. Using nondominant thumb and index finger, scruff mouse.
2. With the other hand, use a caliper to measure perpendicular tumor diameters in mm. *See* **Note 8**.
3. The initial longest measurement, longitudinal, should be noted as the length, while the smallest measurement, transversal, should be noted as the width. If tumor is hard to find, wet area with 70% ethanol to help reveal it.
4. Calculate tumor volume (mm^3) = 0.5 × (length × $width^2$).
5. Measure tumor every other day after CAR T cell injection.

3.6 Dissections

Three weeks (21 days) post tumor injection, tumors and spleens are ready to be removed for analysis. The removal of the tumor and spleen allow a final weight measurement, another metric to compare the tumor growth in addition to tumor volume. It will also allow the analysis of tumor-infiltrating T cells and thus confirmation of successful CAR T cell infusion and trafficking. Of note, it is possible that timeframes proposed here are shortened due to tumors reaching their humane endpoint earlier than anticipated.

1. Euthanize mouse by CO_2 asphyxiation, followed by cervical dislocation, or alternative approved method.
2. Measure final tumor volume with caliper.
3. Measure mouse final weight.
4. Spray mouse with 70% ethanol.
5. Optionally, shave the area around the tumor to minimize hair from getting into sample in downstream tissue processing.
6. Remove tumor and spleen as described below.

3.6.1 Tumor Dissection

1. Using forceps, pull up on the mouse's right flank, creating a "skin tent."
2. With scissors or scalpel, make an incision perpendicular to skin tent.

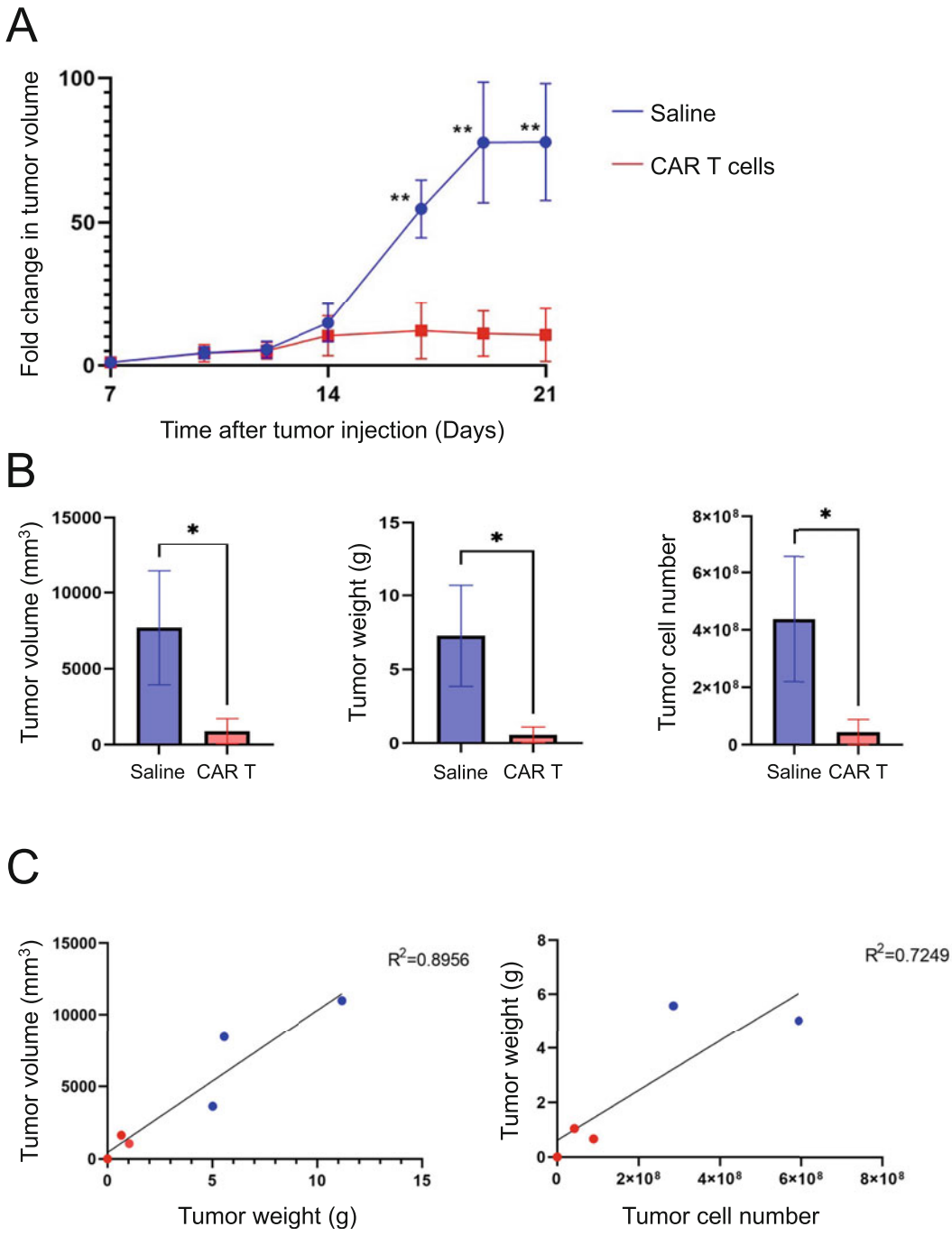

Fig. 3 CAR T cell impact on human solid tumor growth in NSG mice. (**a**) NSG mice were injected with 5×10^6 $CD19^+$ K562 cells. After 7 days, mice with tumor volumes between 50 mm^3 and 150 mm^3 were injected with either saline ($n = 3$) or 1×10^6 human $CD4^+$ T cells and 1×10^6 human $CD8^+$ T cells ($n = 3$) expressing a CD19CAR-2A-GFP construct (Addgene #1359910). Tumor volumes were measured every other day for the following 14 days. Mice were euthanized 21 days after initial tumor subcutaneous (s.c.) injection. The graph displays the fold change in tumor volume relative to initial volume on day 7. (**b**) Final tumor volumes, weights, and cell numbers from experiment described in A. (**c**) Correlation between final tumor volumes (mm^3) and final tumor weights (grams) ($R^2 = 0.8956$) demonstrating that caliper measurements are an accurate representation of tumor growth. Correlation between final tumor weights (grams) and tumor cell number ($R^2 = 0.7249$) demonstrating consistency of the tumor digestion protocol

3. Insert closed scissors into the incision and open the scissors, spreading the incision opening with the dull ends of the scissors.
4. Keep spreading the incision site while gently pulling upward on the skin flap until some of the tumor is exposed.
5. Grab the mouse skin on top of tumor mass with forceps and place a scalpel in between skin and tumor.
6. Gently pull up on the skin as you move scalpel across the tumor edge until the subcutaneous tumor is completely exposed.
7. Move scalpel in between tumor and muscle wall while rotating the mouse to remove the tumor. The muscle wall should not be broken in the process. No hair, scabs, or skin should be left on the tumor and should be consistent across other tumors dissections.
8. Weigh tumor on a 100 × 15 mm Petri dish.
9. Store tumor at 4 °C until further processing.
10. Remove spleen as described below (Subheading 3.6.2).
11. Repeat for each mouse.

3.6.2 Spleen Dissection

1. Using forceps, pull up on mouse's left flank below ribcage, creating a "skin tent."
2. With scissors or scalpel, make an incision perpendicular to skin tent.
3. Insert closed scissors into the incision site and open the scissors, spreading the incision opening with the dull ends of the scissors.
4. Keep spreading the incision site while gently pulling upward on the skin flap until left flank skin is removed.
5. Make a small incision on the muscle wall.
6. Remove the spleen with forceps and trim as much connective and adipose tissue as possible without rupturing the spleen.
7. Weigh spleen on a 60 × 15 mm Petri dish.
8. Add cold RPMI medium.
9. Store at 4 °C until further processing.

3.7 Tissue Processing

After the spleen and tumor are removed, they need to be processed into single-cell suspensions for cell counting and antibody staining. ACK lysis is used to remove red blood cells from the spleen, while collagenase and DNase will be used to digest tumors into single-cell suspensions.

3.7.1 Tumor Tissue

1. Obtain two representative sections of the tumor weighing 0.2–0.3 g for a total of about 0.5 g for the tumor sample. These sections will be digested separately and then combined

once you count the cells. Take note of the total weight for representative section as this is used for the calculation of tumor cell count.

2. With a fresh razor blade, mince each tissue section to a minimum of 1-mm sections inside a 100 × 15 mm Petri dish.
3. Optionally, add 1 mL of cold PBS halfway through to help with mincing.
4. Transfer minced tumor pieces into a 50-mL conical tube.
5. Rinse Petri dish with cold DPBS to collect all tumor pieces and cells.
6. Optionally, run through a 100-μm cell filter to help remove fat, connective tissue, and skin debris. Use a syringe plunger to push minced tumor through the filter.
7. Bring up volume to at least 10 mL of cold DPBS.
8. Centrifuge cells at 500 *g* for 5 min at 4 °C.
9. Decant supernatant. You may observe fat and debris in the supernatant.
10. Briefly vortex cell pellet.
11. Resuspend in 500 μl of serum-free RPMI medium.
12. Transfer resuspended cells into a 1.5-mL microcentrifuge tube.
13. Add 500 μl of 2 mg/mL of PBS Collagenase P to each tube.
14. Add 1 μl DNase I (RNase Free) at 2000 U/mL to each tube.
15. Briefly vortex.
16. Shake samples at 37 °C at 225 rpm for 1.5 h. Longer times can result in the cleaving of surface T cell markers, such as CD4 and CD8α.
17. Transfer processed sample into a 50-mL conical tube and add 10 mL of PBS with 2 mM EDTA to stop collagenase digestion.
18. Combine digested tumor sections from same tumor.
19. Filter solution through a 70-μm filter to a new 50-mL conical tube.
20. Rinse 70-μm filter with an additional 10 mL of DPBS with 2 mM EDTA.
21. Keep on ice.
22. Count cells using 1:1 Trypan Blue. *See* **Note 9**.

3.7.2 Spleen

1. Place a 70-μm filter on a 50-mL conical tube.
2. Pour spleen and RPMI from 60 × 15 mM Petri dish onto 70-μm filter.
3. Grind, and push spleen against the filter using a syringe plunger.

4. Rinse filter with RPMI to obtain a final volume of ca. 10 mL RPMI.
5. Centrifuge at 500 *g* for 5 min at RT.
6. Decant supernatant.
7. Briefly vortex cell pellet.
8. Gently resuspend in 1 mL of ACK lysing buffer.
9. Incubate for 5 min at RT.
10. Stop the reaction by adding 10 mL RPMI.
11. Centrifuge at 500 *g* for 5 min at 4 °C.
12. Resuspend cell pellet in 3 mL cold DPBS.
13. Keep on ice.
14. Count cells with 1:1 Trypan blue. *See* **Note 9**.

3.8 Flow Cytometry

After cells from tumor and spleen have been processed and counted, it is time to phenotype them using flow cytometry. Depending on the antibodies on hand, what reporter gene is incorporated downstream of the CAR gene and flow cytometer used, the panel used may be different from the one described below. In the current protocol, we used GFP as the CAR reporter gene and stained cells for human CD3, CD4, and CD8, as well as with a viability dye (Figs. 4, 5 and 6).

1. Obtain a maximum of 1×10^7 total viable tumor cells and splenocytes for flow cytometry analysis into a 5-mL FACS tube. Take note of how many cells were used to later calculate percentage of tumor-infiltrating leukocytes and engraftment, respectively.
2. Centrifuge at 500 *g* for 5 min at RT.
3. Prepare antibody master mix with DPBS, anti-human CD4 A700 1:100, anti-human CD8 PerCP 1:100, anti-human CD3 PE/Cy7 1:100, and Ghost Viability Dye BV510 1:500. Each sample will be stained with 100 μl antibody master mix. Hence, if staining a total of 12 samples as an example, add 12 μl CD4 A700, 12 μl CD8 PerCP, 12 μl CD3 PE/Cy7, and 2.4 μl Ghost BV510 to 1200 μl DPBS. Store on ice in the dark. *See* **Note 10**.
4. Decant supernatant.
5. Briefly vortex.
6. Add 100 μl antibody master mix to each sample.
7. Briefly vortex and incubate at 4 °C in the dark for 30 min.
8. Wash by adding 500 μl DPBS.
9. Centrifuge at 500 *g* for 5 min at RT.
10. Decant supernatant.

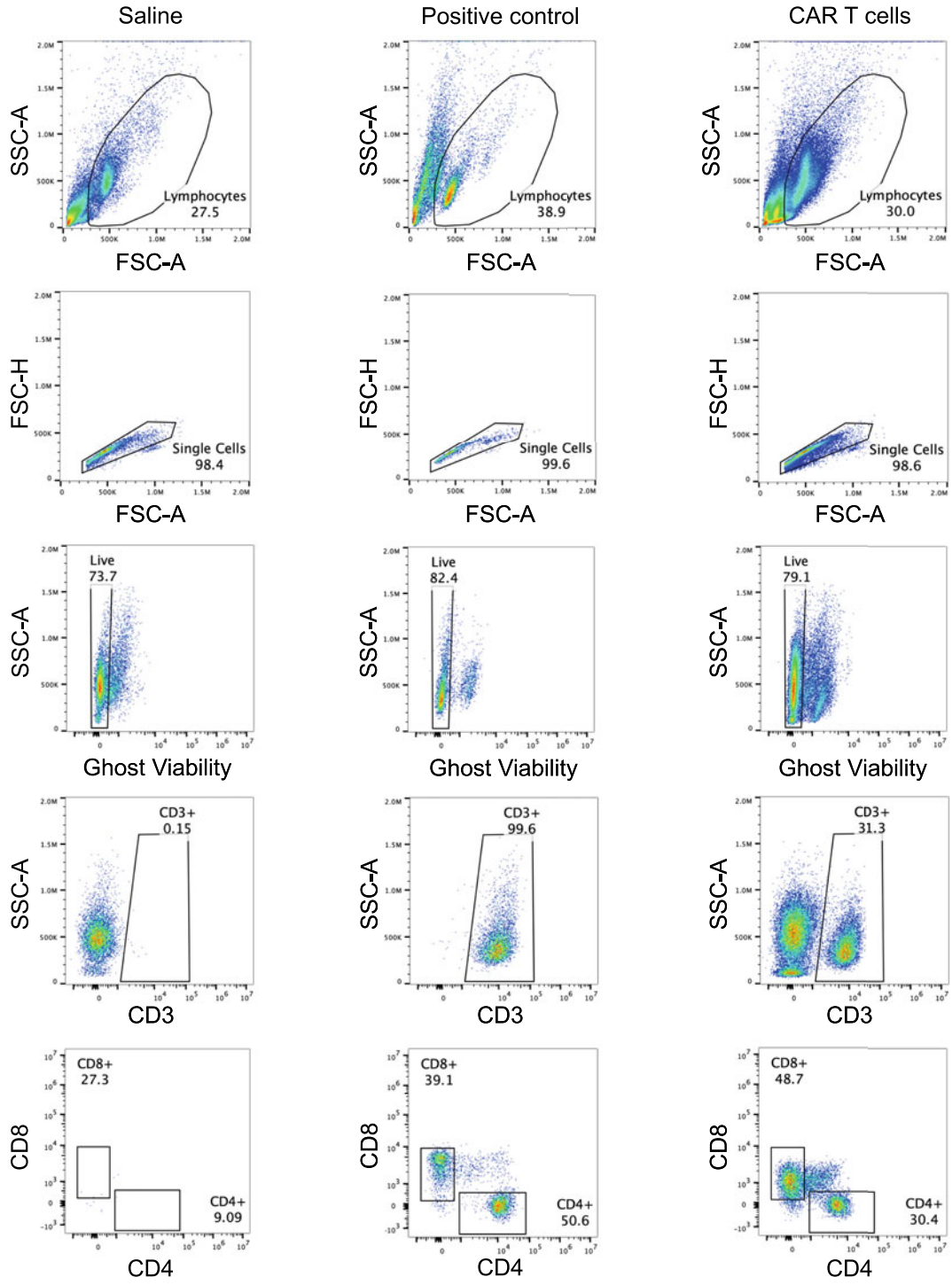

Fig. 4 CAR T cell engraftment in NSG murine spleen. Three weeks (21 days) after tumor injection, single-cell suspensions were collected from saline (DPBS) or CAR T cell-treated mouse spleens and surface stained with anti-human CD4 Alexa700 1:100, anti-human CD8 PerCP 1:100, anti-human CD3 PE/Cy7 1:100, and Ghost Viability Dye BV510 1:500. Positive control was created by adding 1×10^6 CAR^+CD4^+ and CAR^+CD8^+ T cells on top of a nontreated spleen sample

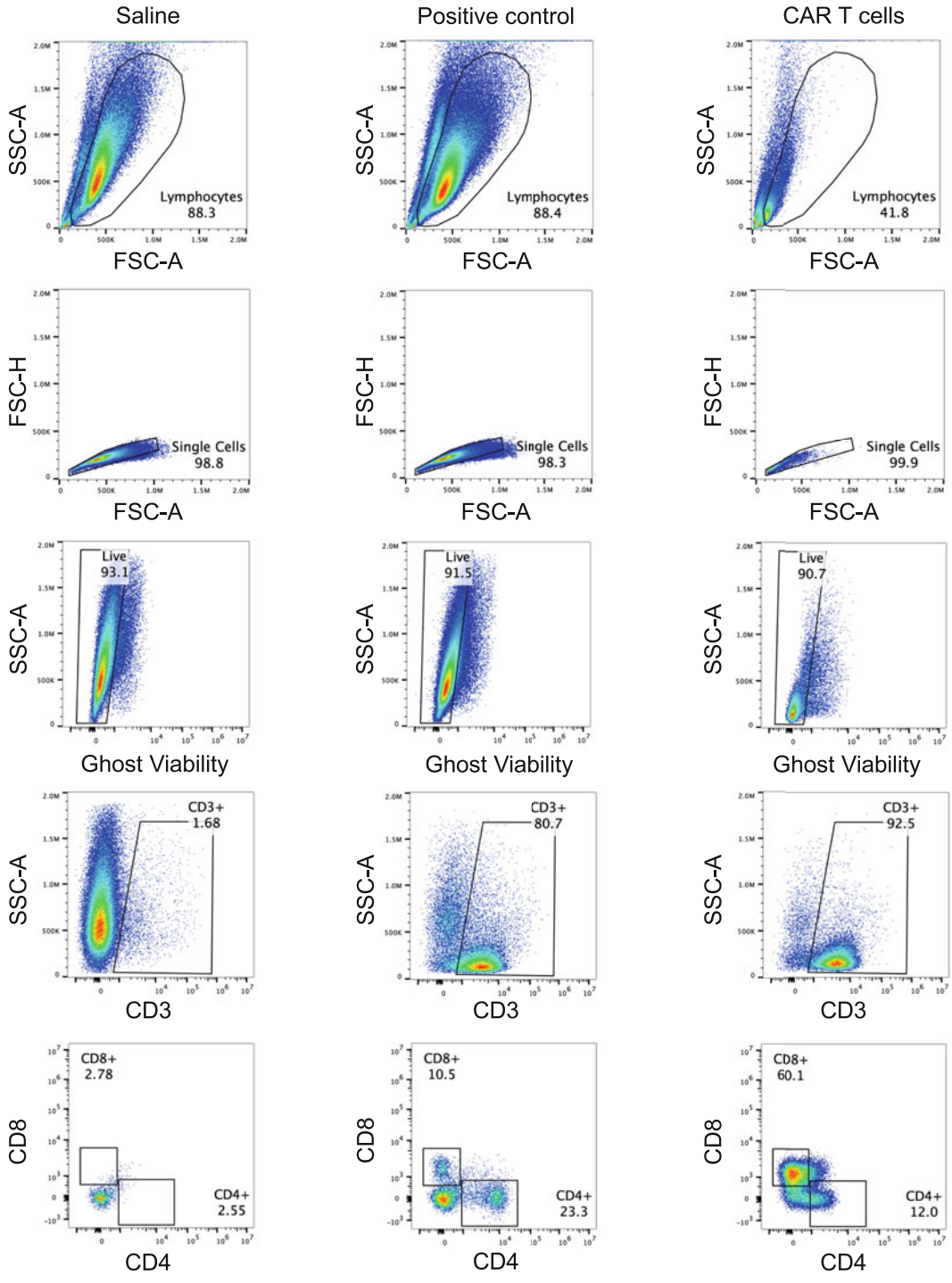

Fig. 5 Tumor-infiltrating T cells in human solid tumors in NSG mice. Three weeks (21 days) after tumor injection, single-cell suspensions were collected from saline (DPBS), or CAR T cell-treated human solid tumors and surface stained with anti-human CD4 Alexa700 1:100, anti-human CD8 PerCP 1:100, anti-human CD3 PE/Cy7 1:100, and Ghost Viability Dye BV510 1:500. Positive control was created by adding 1×10^6 CAR^+CD4^+ and CAR^+CD8^+ T cells on top of a nontreated tumor sample

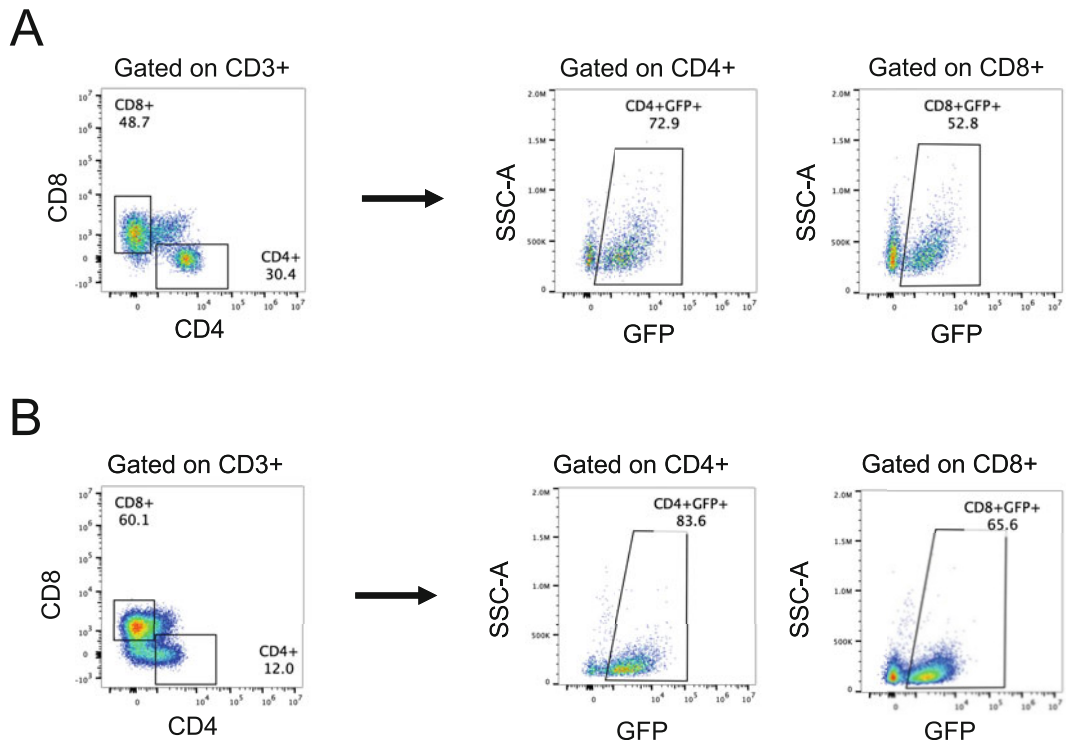

Fig. 6 Detecting CAR T cells in the spleen and tumor in NSG mice. (**a**) CAR T cells (CAR^+CD4^+ and CAR^+CD8^+ T cells) were detected in the spleen 2 weeks after intravenous T cell injection by assessing the expression of the CD19CAR-2A-GFP construct (Addgene #1359910) reporter gene, GFP, within viable human $CD3^+CD4^+$ and viable human $CD3^+CD8^+$ cells. (**b**) Analogously to (**a**), CAR T cells were detected in the tumor 2 weeks after intravenous T cell injection by assessing GFP expression

11. Resuspend the cell pellet in 300 μl DPBS.
12. Keep tubes on ice in the dark.
13. Analyze by flow cytometry. Filter samples through 40-μm filter cap into flow tubes immediately before reading to guarantee single cell suspensions. *See* **Note 11**.
14. Using total tumor cell number and percentage of CAR^+ T cells in tumor sample, calculate the number of tumor-infiltrating CAR^+ T cells per tumor.

4 Notes

1. If using an automated cell counter, e.g., Bio-Rad TC20 Automated Cell Counter, adjust cell count to this 200-fold cell dilution by multiplying the result by 100, as most cell counters assume a 1:1 dilution.
2. If T cells are not elongated and clustering by 24 h, then activation and/or isolation was not performed properly.

3. If lentivirus coding for the CAR or antigen of choice is to be produced in lab rather than purchased, please refer to *Zimmerman et al., this issue*, for methods on producing and titrating lentivirus.
4. $CD8^+$ T cells have a lower transduction efficiency with lentivirus than $CD4^+$ T cells at the same MOI. Therefore, transduce 2–3 times more $CD8^+$ T cells than $CD4^+$ T cells to have enough CAR^+ cells for in vivo experiments.
5. Irradiated antigen-expressing K562 can be used to stimulate and expand CAR T cells in vitro; please see *Zimmerman et al., this issue*.
6. On the day of subcutaneous tumor injection, i.e., 7 days before CAR T cell intravenous injection, ensure antigen-expressing K562 cells are in the logarithmic growth phase by harvesting cells from flasks 50–80% confluent and at least 90% viable.
7. Due to the variability of tumor growth, it is recommended to inject more mice than the intended number of mice to receive CAR T cells. This will ensure there are enough mice with tumors between 50 mm^3 and 150 mm^3 on the day of CAR T cell injection.
8. As it is crucial to be consistent with the tumor measurements each time, it is recommended that the same person make all measurements across all days and take note of how tight the caliber is placed against the tumor for maximum consistency.
9. If tumor and spleen processing need to be carried out over 2 days, then this is a good place to stop. If this is the case, then cells should be given in RPMI10 in **step 17** instead of DPBS with 2 mM EDTA to provide nutrients to single-cell suspension. Place single-cell suspension at 4 °C overnight until antibody staining for flow cytometry. Note that a 30–50% loss of total cells is expected if cells are kept overnight instead of analyzed on the same day.
10. Multicolor flow cytometry requires preparation of single-color controls for the cytometer to perform compensation, i.e., correct for spectral overlap between different channels. Hence, for the described panel, prepare the following single-color controls: unstained, A700, PerCP, PE/Cy7, BV510, and GFP.
11. Take into consideration that CD4 and CD8α may be downregulated due to collagenase treatment [12]. These effects can be exacerbated depending on incubation time and type of collagenase used.

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Chapter 17

Nano-optogenetic CAR-T Cell Immunotherapy

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Abstract

Chimeric antigen receptor (CAR)-T cell immunotherapy emerges as an effective cancer treatment. However, significant safety concerns remain, such as cytokine release syndrome (CRS) and "on-target, off-tumor" cytotoxicity, due to a lack of precise control over conventional CAR-T cell activity. To address this issue, a nano-optogenetic approach has been developed to enable spatiotemporal control of CAR-T cell activity. This system is comprised of synthetic light-sensitive CAR-T cells and upconversion nanoparticles acting as an in situ nanotransducer, allowing near-infrared light to wirelessly control CAR-T cell immunotherapy.

Key words Nano-optogenetics, CAR T cell, Cancer immunotherapy, Synthetic immunology, Wireless control, Near-infrared light, Upconversion nanoparticles

1 Introduction

Chimeric antigen receptor (CAR)-T cell immunotherapy has shown promising potential in eradicating tumors [1–4]. These synthetic receptors are engineered into the plasma membrane of effector T-cells, allowing them to engage with specific tumor antigens independently of the major histocompatibility complex. This recognition triggers the activation of engineered T-cells, which can then perform their function of killing tumors. However, despite its success in treating cancer, CAR-T cell therapy still presents safety challenges due to the lack of precise control over T-cell activity in terms of dose, location, and timing. Examples of these challenges are cytokine release syndrome (CRS) and "on-target, off-tumor" cytotoxicity [5, 6], such as B-cell aplasia. As a result, it is crucial to develop intelligent CAR-T cell-based therapies that offer precise control over therapeutic activities in a spatiotemporal manner.

Here, we developed a nano-optogenetic approach [7–10] which enables spatiotemporal control of the CAR-T cell immunotherapy wirelessly by the near-infrared light (NIR). We have engineered light sensitivity into the chimeric antigen receptors, creating

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light-switchable CAR (LiCAR) T-cells that can selectively produce antitumor immune responses in the presence of both tumor antigen and light. To demonstrate the feasibility of wireless optogenetic intervention in vivo, we combined LiCAR with surgically removable upconversion nanoplates (UCNPs) that have enhanced NIR-to-blue upconversion luminescence. The UCNPs act as miniature light transducers, allowing inducible activation of CAR-T cells in living animals upon stimulation with deep tissue-penetrating NIR light. This NIR light-tunable nano-optogenetic immunomodulation platform enables spatiotemporal control of CAR-T-cell-mediated cytotoxicity against both hematological malignancies and solid tumors, with tailored doses and duration.

2 Materials

2.1 Media

1. T-cell medium R10 (RPMI 1640 + 10% FBS + supplements): 500 mL RPMI, 50 mL heat-inactivated FBS, 5 mL sodium pyruvate, 5 mL NEAA, 20 mM HEPES, 5 mL Pen + Strep + glutamine.
2. DMEM medium for 293 T cells: 500 mL DMEM, 50 mL HI FBS, 5 mL NEAA, 5 mL sodium pyruvate, 5 mL Pen + Strep + glutamine.
3. Cell freezing medium: 90% FBS, 10% DMSO.
4. DMEM 10% FBS medium: 90% DMEM, 10% FBS.
5. Leukopak.
6. Opti-MEM medium.

2.2 Buffers

1. FACS buffer (PBS + 2 mM EDTA +0.5% BSA): 500 mL PBS, pH 7.4 w/o Ca/Mg, 25 mL MACS BSA stock solution (commercial stock is at 10%), 2 mL 0.5 M EDTA.
2. Plate blocking buffer (PBS + 2% BSA): 20 mL PBS, pH 7.4 w/o Ca/Mg, 5 mL MACS BSA stock solution (commercial stock is at 10%).
3. 2% BSA blocking solution: 5 mL 10% BSA + 20 mL PBS.

2.3 Antibodies, Reagents, and Kits

1. EasySep™ Human T Cell Isolation Kit: EasySep buffer, isolation cocktail, RapidSpheres. Purchase from STEMCELL Technologies.
2. Easy50 magnet. Purchase from STEMCELL Technologies.
3. Anti-CD3 monoclonal antibody.
4. Anti-CD4 monoclonal antibody.
5. Anti-CD8 monoclonal antibody.

6. Lipofectamine™ 2000 Transfection Reagent. Purchase from Invitrogen.
7. MSGV1-CAR retrovirus vector.
8. Plasmid RD114.
9. Plasmid Gag/pol.
10. Human IL-2 IS (interleukin 2 "Improved Sequence"). Purchase from Miltenyi Biotec.
11. T Cell TransAct. Purchase from Miltenyi Biotec.
12. Poly-D-Lysine.
13. Trypsin.
14. RetroNectin. Purchase from Thermo Fisher.
15. Streptavidin.
16. EZ-Link Sulfo-NHS-Biotin. Purchase from Thermo Fisher.
17. APC-conjugated streptavidin.
18. Liberase™ TL (Thermolysin Low) kit.
19. ACK Lysing Buffer. Purchase from Lonza Group Ltd.
20. Corning Matrigel Matrix.

2.4 Chemicals

1. CF_3COONa.
2. $Yb(CF_3COO)_3$.
3. $Tm(CF_3COO)_3$.
4. Oleic acid.
5. Oleylamine.
6. 1-Octadecene.
7. Ethanol.
8. Hexane.
9. Igepal CO-520.
10. Tetraethoxysilane.
11. Ammonia.
12. Dimethylformamide.
13. Nitrosonium tetrafluoroborate.
14. Poly(acrylic acid) (PAA).
15. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).
16. N-Hydroxysuccinimide (NHS).

3 Methods

Carry out all procedures at room temperature (RT) unless otherwise specified.

3.1 Isolation of Human Pan T-Cells from PBMC

1. Transfer 25 mL of leukopak to 50-mL conical tubes.
2. Add 25 mL EasySep buffer. Spin at 500 g for 10 min and aspirate supernatant.
3. From above, resuspend cells to 5×10^7 cells/mL in EasySep buffer. Make sure the volume is between 10 and 45 mL.
4. Add 50 μL of isolation cocktail per mL of cells.
5. Incubate for 5 min at room temperature.
6. Vortex RapidSpheres for 30 s and add 40 μL/mL sample.
7. If total volume is <20 mL, top up to 25 mL with EasySep buffer. If total volume >20 mL, top up to 50 mL with EasySep buffer.
8. Gently mix up and down 2–3 times.
9. Place tube onto Easy50 magnet without lid and incubate for 10 min at RT.
10. Carefully pipette enriched cell suspension to a new 50-mL tube.
11. Place tube back onto Easy50 magnet without lid and incubate for 5 min at RT.
12. Carefully pipette enriched cell suspension to a new 50-mL tube.
13. Dilute to 50 mL with EasySep buffer and transfer to new 50-mL conical, filtering through a 70-μm cell strainer.
14. Count cells and use immediately or freeze down in cell freezing medium. Save some cells for purification analysis.
15. Verify pan T purity by flow cytometry as described from **steps 16–22**.
16. Transfer 100 μL of cell sample to a 96-well plate. Add 100 μL FACS buffer
17. Centrifuge at 500 g for 5 min and aspirate supernatant
18. Prepare antibody staining solution including anti-CD3, anti-CD4, and anti-CD8 monoclonal antibody.
19. Resuspend cells in 50 μL stain. Incubate 30 min on ice or at 4 °C.
20. Add 150 μL FACS buffer and centrifuge at 500 g for 5 min.
21. Resuspend cells in 200 μL FACS buffer.
22. Run sample on a flow cytometer (200 μL/min, 30 K events).

3.2 Transduction of CAR Constructs into T Cells

1. On day-1, seed 293 T for transfection as described from **steps 2–10**.
2. Coat each well/flask with 0.25× working volume of poly-D-Lysine for at least 1 min (500 μL for 6-well plates).
3. Aspirate and let dry for at least 10 min.
4. Trypsinize 293 T for 5 min at 37 °C. Add the same volume of DMEM 10% FBS into trypsinized flash.
5. Centrifuge at 400 g for 5 min.
6. Aspirate supernatant and resuspend cells in 10 mL DMEM 10% FBS.
7. Count cells.
8. Resuspend cells to 0.4×10^6 cells/mL DMEM 10% FBS.
9. If transfections are to be done at 6 well plate scale, add 2 mL per well (0.8×10^6 cells total).
10. Incubate at 37 °C, 5% CO_2.
11. On day 0, perform the cell transfection as described from **steps 12–23**.
12. Check that 293 T are 70–90% confluent prior to transfection.
13. Warm Opti-MEM to room temperature.
14. Calculate the required components of transfection master mixes at 6-well plate scale. Each well of 6-well plate finally receives 10 μL Lipofectamine 2000, 2.5 μg total plasmid DNA at a ratio 2:1:1 for MSGV1-CAR retrovirus vector/RD114/Gag/pol, and up to 500 μL Opti-MEM.
15. Prepare helper plasmid master mix including 0.625 μg RD114, 0.625 μg Gag/pol, and add Opti-MEM up to 250 μL per reaction.
16. Transfer 250 μL of helper plasmid master mix into Eppendorf tubes.
17. Add 1.25 μg MSGV1-CAR plasmid per tube.
18. Incubate for 5 min at room temperature.
19. Prepare Lipofectamine master mix which include10 μL Lipofectamine 2000 and add Opti-MEM up to 250 μL per reaction.
20. Add 250 μL of Lipofectamine master mix to each transfection tube containing MSGV1-CAR and helper plasmid and mix by pipetting.
21. Incubate for 20 min at room temperature.
22. Carefully drip 500 μL of each transfection mix to corresponding plates containing 293 T.
23. Incubate at 37 °C, 5% CO_2 overnight.

24. On day 0, perform the activation of human pan T-cells as described from **steps 25–32**.
25. Thaw aliquot of human pan T-cells isolated from PBMCs in 37 °C water bath.
26. Transfer cells to 50-mL conical tube and slowly dilute with 9 mL R10.
27. Centrifuge at 400 g for 5 min.
28. Aspirate supernatant and resuspend cells in 10 mL R10.
29. Count cells.
30. Dilute (or concentrate) cells to 10^6 cells/mL in R10 supplemented with 100 IU/mL human IL-2 IS.
31. Add 1:100 TransAct (v/v) directly to cells and plate into 6-well plate (5 mL max per well).
32. Incubate at 37 °C, 5% CO_2.
33. On day 1, change medium for transfected plate as described from **steps 34–36**. Treat all tips, media, and plates as virus-contaminated from this point forward. Keep a beaker of bleach in a hood to decontaminate media and pipettes before disposal.
34. To transfected 293 T plate, remove media after 18–20 h post-transfection.
35. Gently add fresh RPMI, 10% FBS to the side of the well.
36. Incubate at 37°C, 5% CO_2 overnight.
37. On day 1, prepare RetroNectin plate as described from **steps 38–39**.
38. Add 150 μL 1 mg/mL RetroNectin to 12 mL PBS to prepare 12.5 μg/mL RetroNectin coating solution.
39. Add 500 μL RetroNectin coating solution per well of a non-TC-treated 24-well plate. Coat at 4 °C overnight.
40. On day 2, harvest retroviral supernatant and transduction as described from **steps 41–54**.
41. Harvest and filter retroviral supernatant through 0.45-μm syringe filter. Use fresh or freeze at −80 °C.
42. Add 500 μL 2% BSA blocking solution per well of a RetroNectin-coated 24-well plate.
43. Block for 10 min at room temperature.
44. Aspirate block solution.
45. Rinse wells with 500 μL PBS.
46. Aspirate PBS.
47. Add 1000 μL retroviral supernatant per well of a RetroNectin-coated 24-well plate.

48. Spin virus on RetroNectin-coated plate for 2 h at room temperature at 3000 g.
49. Remove viral supernatant from the plate after the spin is completed.
50. Rinse wells with 500 μL PBS.
51. Aspirate PBS.
52. Add 500 μL RPMI, 10% FBS, and 100 IU/mL IL-2 to each well.
53. Add 0.2 million activated T-cells per well.
54. Incubate at 37 °C, 5% CO_2.
55. On day 3, to each well, add 1 mL RPMI, 10% FBS, and 150 IU/mL IL-2 Incubate at 37°C, 5% CO_2.
56. On day 6, perform the cell expansion as described from **steps 57–59**.
57. Transfer cells to 24-well G-Rex for expansion. Top up the volume to 6.5 mL with fresh RPMI, 10% FBS, supplemented with 100 IU/mL IL-2.
58. Feed cells with IL-2 every 2 days; new medium every 3–4 days.
59. Check transduction efficiency using a flow cytometer based on a fluorescence tag.
60. On day 14, collect CAR-T cell as described from **steps 61–64**.
61. CAR-T cells are harvested by centrifuging at 400 g for 5 min.
62. Aspirate supernatant and resuspend cells in 10 mL PBS.
63. Count cells.
64. Centrifuge and concentrate cells to 2×10^7 cells/mL in PBS. CAR-T cells are kept in ice until inject into mouse.

3.3 UCNP Synthesis

1. Synthesize β-$NaYbF_4$:0.5%Tm@$NaYF_4$ core-shell UCNPs as described from **steps 2–15**.
2. CF_3COONa (0.50 mmoL), $Yb(CF_3COO)_3$ (0.4975 mmoL), and $Tm(CF_3COO)_3$ (0.0025 mmoL) precursors were mixed with oleic acid (5 mmoL), oleylamine (5 mmoL), and 1-octadecene (10 mmoL) in a two-neck round-bottom flask.
3. The mixture was heated to 110 °C to form a transparent solution followed by 10 min of degassing.
4. Then the mixture was heated to 300 °C at a rate of 15 °C/min under dry argon flow and maintained at 300 °C for 30 min to form the α-$NaYbF_4$:0.5%Tm intermediate UCNPs.
5. After the mixture cooled down to room temperature, the α-$NaYbF_4$:0.5%Tm intermediate UCNPs were collected by centrifugal washing with excessive ethanol (7500 g, 10 min).

6. In the second step, the α-$NaYbF_4$:0.5%Tm intermediate UCNPs were redispersed into oleic acid (10 mmoL) and 1-octadecene (10 mmoL) together with CF_3COONa (0.5 mmoL) in a new two-neck round-bottom flask.
7. After degassing at 110 °C for 10 min, this flask was heated to 325 °C at a rate of 15 °C/min under dry argon flow and maintained at 325 °C for 30 min to complete the phase transfer from α to β.
8. After the mixture cooled to room temperature, the β-$NaYbF_4$:0.5%Tm core UCNPs were collected by precipitation with an equal volume of ethanol followed by centrifugation (7500 g, 10 min).
9. The β-$NaYbF_4$:0.5%Tm core UCNPs were stored in hexane (10 mL).
10. In the third step, the as-synthesized β-$NaYbF_4$:0.5%Tm core UCNPs served as cores for the epitaxial growth of core–shell UCNPs.
11. The hexane stock solution of β-$NaYbF_4$:0.5%Tm core UCNPs was transferred into a two-neck round-bottom flask, and the hexane was sequentially evaporated by heating.
12. CF_3COONa (0.25 mmol) and $Y(CF_3COO)_3$ (0.25 mmoL) were introduced as UCNP shell precursors with oleic acid (10 mmoL) and 1-octadecene (10 mmoL).
13. After 10 min of degassing at 110 °C, the flask was heated to 325 °C at a rate of 15 °C/min under dry argon flow and maintained at 325 °C for 30 min to complete the shell crystal growth.
14. After the mixture cooled to room temperature, the β-$NaYbF_4$:0.5%Tm@$NaYF_4$ core-shell UCNPs were collected by precipitation with an equal volume of ethanol followed by centrifugation (7500 g, 10 min).
15. β-$NaYbF_4$:0.5%Tm@$NaYF_4$ core–shell UCNPs were stored in hexane (10 mL).
16. Synthesize silica coated core–shell UCNPs as described from **steps 17–22**.
17. 4 mL of the core-shell UCNP hexane solution was added to 21 mL hexane in a 50-mL one-neck round bottom flask.
18. 1.5 mL of Igepal CO-520 was added to the solution which was kept in a water bath while sonicated for 2 min.
19. 160 μL of ammonia was added to the solution.
20. After 30 min of stirring, 80 μL of TEOS (Tetraethoxysilane) was added to the solution.

21. After 2 days of stirring, the silica-coated core–shell UCNPs were collected by precipitation with an equal volume of ethanol, followed by centrifugation (7500 g, 10 min) for three times.
22. The silica-coated core–shell UCNPs were stored in water (20 mL) for further use.
23. Synthesize streptavidin-conjugated UCNPs (Stv-UCNPs) as described from **steps 24–33**.
24. UCNPs in hexane (1 mL) were added to a dimethylformamide (DMF) solution (5 mL) containing nitrosonium tetrafluoroborate (0.20 g).
25. The mixture was stirred for 2 h and then allowed to stand for 5 min.
26. The UCNPs capped by BF_4^-dispersed at the bottom DMF layer were taken out and precipitated with isopropanol (5 mL) by centrifuging at 11000 g for 15 min.
27. The washing steps were repeated for two additional cycles with 5 mL of isopropanol and 5 mL DMF.
28. The UCNPs were dispersed in 5 mL DMF solution containing PAA (50 mg) and coated onto UCNPs after overnight incubation with constant stirring.
29. The UCNPs-PAA were washed for three cycles by deionized (DI) water and centrifugation at 11,000 g for 15 min.
30. The UCNPs were dispersed in 5 mL DI water and activated by EDC (50 mg) and NHS (10 mg) to form succinimidyl ester for 2 h, followed by washing with DI water and centrifugation at 11,000 g for 15 min.
31. After redispersing the UCNPs in 5 mL DI water, streptavidin (150 μg) was added and the mixture was stirred at room temperature for 4 additional hours.
32. The streptavidin-UCNPs (Stv-UCNPs) were collected, washed three times by DI water and centrifuged at 11,000 g for 15 min.
33. Stv-UCNPs were suspended in PBS at a concentration of 1.5 mg/mL for further functional studies.

3.4 T-Cell Biotinylation and Coupling with Stv-UCNPs

1. Jurkat or human $CD8^+$ T-cells expressing WT CAR or LiCAR, as well as Jurkat T-cells as control, were washed three times in cold PBS to deplete residual free amine-containing buffers.
2. Cells were then suspended at a concentration of 2.5×10^7 cells/mL.
3. 200 μL of 10 mM EZ-Link Sulfo-NHS-Biotin was added to 1 mL of cell suspension (Fig. 1).

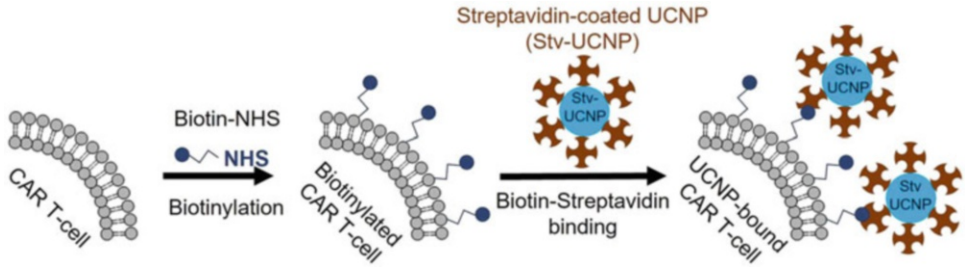

Fig. 1 Schematic demonstrating the surface biotinylation process of engineered CAR-T cells and their linkage with streptavidin-coated upconversion nanoparticles (UCNPs). Reproduced with the permission [9]. (Copyright 2021, Springer Nature)

4. The mixture was incubated at 4 °C for 30 min.
5. The cells were washed three times with 100 mM glycine in PBS.
6. Biotinylated cells were further incubated with APC-conjugated streptavidin to evaluate the biotinylation efficiency by flow cytometry.
7. To prepare for Stv-UCNP-CAR T-cell mixture, 0.1 mL Stv-UCNPs (1.5 mg/mL) was mixed with 1×10^7 biotinylated $hCD8^+$ T-cells expressing WT CAR or LiCAR and then incubated at 4 °C for 30 min.
8. Unbound St-UCNPs were washed away by centrifuging the mixture at 700 g for 5 min.
9. UCNP-coupled CAR-T cells were re-suspended in PBS and administered into mice via tail vein injection.

3.5 Mouse Model

1. Develop syngeneic mouse model of melanoma as described from **steps 2–15**.
2. On day 0, inoculate 6–12-week-old C57BL/6 J mice (either sex) with $2.5–5 \times 10^5$ B16-OVA and B16-OVA-hCD19 or B16-OVA-mCD19 cells depending on each experiment via intradermal injection.
3. Measure the size of the tumors with a digital caliper every day, and calculate the tumor area in square millimeters (length x width) when the tumors become visible.
4. Allow the tumors to grow for 8 additional days after inoculation.
5. On day 9, co-inject 2×10^6 mouse CD8 T-cells expressing CAR constructs and 150 μg of UCNP into each tumor.
6. From day 10, subject LiCAR-treated mice (hLiCAR or mLiCAR) to pulsed near-infrared light treatment (980 nm at a power density of 250 mW/cm^2; pulse, 20 s ON; 5 min OFF for 2 h per day).

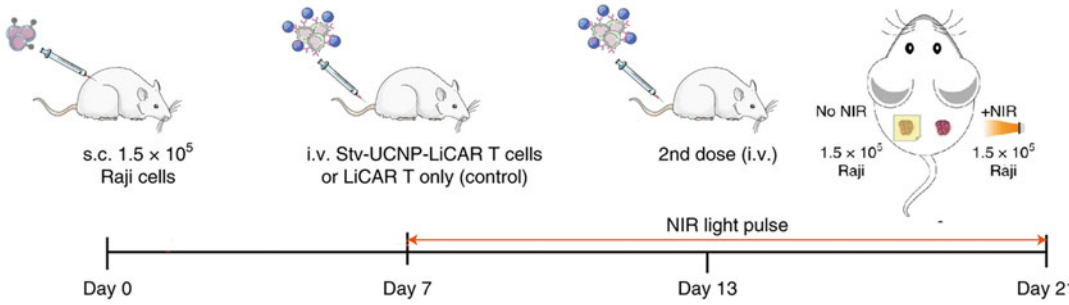

Fig. 2 Schematic of the experimental procedures. 1.5×10^5 Raji cells into each flank of scid-beige mice at day 0, followed by intravenous treatment with either 1×10^7 Stv-UCNP-coupled LiCAR-hCD8$^+$ T-cells (Stv-UCNP-LiCAR) or LiCAR T-cells (control) at day 7. From day 7 to day 21, the mice received pulsed near-infrared (NIR) light stimulation and a second dose of Stv-UCNP-LiCAR or LiCAR T-cells. On day 21, the mice were euthanized to obtain the tumors for phenotypic analyses. Reproduced with the permission [9]. (Copyright 2021, Springer Nature)

7. On day 16–19, collect tumors from euthanized mice.
8. For the analysis of hLiCAR T-cells residing within the tumors, collect tumors, perfuse in PBS, cut into small pieces and enzymatically digest with 5 μg/mL of Liberase TL for 1 h at 37 °C.
9. Filter tumor cells using a 100-μm cell strainer.
10. Wash cells twice in PBS to remove cell debris and resuspend in FACS buffer.
11. Determine the number of adoptively transferred hLiCAR T-cells by detecting hLiCAR fluorescence protein using the LSRII flow cytometer.
12. Analyze the data using FACSDiva8.0 and FlowJo software gated on the GFP$^+$/mCh$^+$ population.
13. For the analysis of hLiCAR T-cells residing within the spleen or blood, isolate spleen cells by crushing the spleen on the strainer as described above while collecting blood cells from the retro-orbital sinus by glass capillary from anesthetized mice.
14. Treat spleen and blood cells with ACK lysis buffer to remove red blood cells.
15. Wash spleen and blood cells twice before performing flow cytometry analyses.
16. Develop mouse model of lymphoma as described from **steps 17–21**.
17. On day 0, 8–12-week-old C.B-Igh-1b/GbmsTac-Prkdcscid-Lystbg N7 mice were inoculated intradermally on both flanks with the mixture of 1.5×10^5 CD19$^+$ Raji cells and Matrigel matrix at a volume ratio of 1:1 (total volume 100 μL per side).

18. Tumor size was measured with a digital caliper every 1–2 days, and the tumor area was calculated in square millimeters (length × width).
19. After 7 days, systemic administration Stv-UCNP-LiCAR T-cells. 1×10^7 engineered T-cells were injected via tail vein to Raji lymphoma mouse models with 2 weekly doses (Fig. 2).
20. All treated mice were subjected to pulsed NIR light stimulation for 13–14 days (980 nm at a power density of 250 mW/cm^2; 20 sec ON + 5 min OFF; 2 h/day; NIR groups), or shielded from the NIR light source (dark groups).
21. On day 20 or 21, mice were euthanized for tumor isolation and phenotypic analyses.

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Chapter 18

A Nonviral *piggyBac* Transposon-Mediated Method to Generate Large-Scale CAR-NK Cells from Human Peripheral Blood Primary NK Cells

Zhicheng Du, Tianzhi Zhao, Xianjin Chen, Shijun Zha, and Shu Wang

Abstract

With the inherent antitumor function and unique "off-the-shelf" potential, genetically engineered human natural killer (NK) cells with chimeric antigen receptors (CARs) bear great promise for the treatment of multiple hematological malignancies and solid tumors. Current methods of producing large-scale CAR-NK cells mainly rely on mRNA transfection and viral vector transduction. However, mRNA CAR-NK cells were not stable in CAR expression while viral vector transduction mostly ended up with low efficiency. In this chapter, we described an optimized protocol to generate CAR-NK cells by using the *piggyBac* transposon system via electroporation and to further expand these engineered CAR-NK cells in a large scale together with artificial antigen-presenting feeder cells. This method can stably engineer human primary NK cells with high efficiency and supply sufficient scale of engineered CAR-NK cells for the future possible clinical applications.

Key words Non-viral, Piggybac transposon system, NKG2D CAR-NK cells, K562 APCs, Cancer immunotherapy

1 Introduction

Adoptive cell transfer of natural killer (NK) cells has become one of the most prominent immunotherapies due to the innate antitumor characteristics and alloreactive potential of NK cells. Allogeneic NK cells, as a perfect candidate in "off-the-shelf" therapies, are typically collected and derived from donors' peripheral blood mononuclear cells (PBMCs) or cord blood mononuclear cells (CBMNCs). They have been tested in multiple clinical trials for cancer treatment [1], in which cell production costs could be significantly reduced compared to autologous cell therapy. As the results of reported clinical trials, allogeneic NK cells were shown to be safe and comparably effective against hematological malignancies, but limited efficacy on solid tumors [1].

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To further improve the therapeutic efficacy in malignancy treatments, especially for solid tumors [2, 3], a chimeric antigen receptor (CAR) was introduced to enhance the antitumor activity of NK cells. CAR is an artificial protein expressed on the cell membrane, which can recognize specific antigens and trigger subsequent intracellular antitumor signaling pathways in the equipped immune cells [4]. CAR was initially implemented on T cells, whereas studies on CAR-engineered NK cells are widely performed in the scenario of treating solid tumors [5]. As reported by Xiao et al. (2019), three patients treated with NKG2D CAR-NK cells achieved satisfied safety profiles, demonstrating significant antitumor effects on treating metastatic colorectal cancer with CAR-NK therapy [3].

In clinical settings, large-scale preparation and stable genetic modification of primary NK cells have nevertheless become a key obstacle in CAR-NK cell therapy. Nowadays, in the majority of current studies, CAR-NK cells are prepared by either viral vector transduction or nonviral mRNA electroporation [3]. However, there are limitations to these methods. On the one hand, the nonviral mRNA electroporation method cannot guarantee the long-term stable expression of CAR. Therefore, multiple injections are usually required for persistent therapeutic effects in clinical usage, which hinders the application of mRNA CAR-NK. On the other hand, although viral vector transduction has been broadly validated in CAR-T cell manufacturing, NK cells are comparably notorious to T cells in genetic modification. With a limited surface expression of viral-associated receptors and intrinsic antiviral functions [6, 7], NK cells are difficult to be transduced by most viral vectors, which highly constrains the efficiency of CAR-NK production. In addition, viral vector preparation is also time- and labor-consuming, challenging in lot-to-lot stability, and complicated in compliance with good manufacturing practice (GMP), which incurs remarkable costs in its large-scale production. In this case, a nonviral stable transfection system is required in CAR-NK manufacturing, especially for clinical treatments.

The piggyBac (PB) transposon system, as a nonviral genetic modification method, has been well established for CAR-T-based immunotherapy [8–11]. Typically, there are two components in this system: a DNA vector, which contains the CAR constructs with flanked terminal inverted repeats (TIRs) [12], and a transposase, which mediates the gene transfer between vectors and host chromosomes. In terms of NK cell modification, the PB transposon system has the potential to generate stable CAR-expressing NK cells with low immunogenicity, enhanced safety profile, and reduced production cost compared to viral vector transduction [12]. Primary NK cells with CAR modification were recently reported on bioRxiv using a Tc Buster transposon system [13], while PB transposon system–mediated CAR engineering was only

studied on NK-92 cell line [14] and barely optimized for large-scale primary CAR-NK cell preparation.

In this chapter, we presented a protocol for the generation and expansion of large-scale NKG2D CAR-NK cells from human peripheral blood primary NK cells with the nonviral PB transposon system and artificial antigen-presenting cells (aAPCs) (Fig. 1) [15]. Theoretically, this method could also be applied in the generation and expansion of NK cells with any other antigen-specific CAR constructs by designing the PB transposon plasmid and aAPCs accordingly.

2 Materials

2.1 Cell Culture

1. The healthy donors' leukocyte reduction system cones are available from the Health Sciences Authority of Singapore.
2. The K562 aAPCs with membrane-bound IL-15, IL-21, and 41BBL are gamma-irradiated at 100 Gy to halt their proliferation.
3. NK cell culture: AIM-V (Invitrogen, Carlsbad, CA) supplemented with 5% AB serum (Valley Biomedical, Winchester, VA), 50 IU/mL IL-2 (PeproTech, Rocky Hill, NJ), 2.5 μg/mL DNase, T75 Nunc EasYFlask 75 cm^2 with filter cap.
4. K562 aAPC culture: IMDM supplemented with 10% fetal bovine serum (FBS), Nunc EasYFlask 175 cm^2 with filter cap.

2.2 Cell Isolation

1. Ficoll-Paque™ Plus.
2. 1× phosphate-buffered saline (PBS).
3. FBS.
4. AB serum.
5. Freezing media: AB serum +10% DMSO.
6. NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.3 Electroporation

1. 4D-Nucleofector™ system (Lonza, Basel, Switzerland).
2. Nucleocuvette™ Vessel (Lonza).
3. P3 Primary Cell Nucleofector ™ Solution (Lonza).
4. Opti-MEM.

2.4 Phenotyping

1. BD Accuri™ C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).
2. All the antibodies required have been summarized in Table 1.

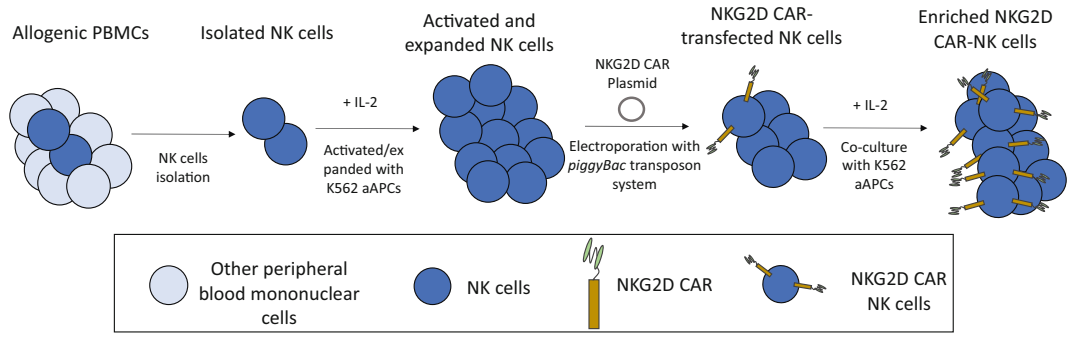

Fig. 1 Schematic diagram for NKG2D CAR-NK cell generation

Table 1
Antibodies for NK cells and CAR-NK cells' flow cytometry analysis

	Company	Clone
THE™ NWSHPQFEK tag FITC	Genescript	5A9F9
CD3 PE	Mitenyi Biotec	BW264/56
CD56 APC	Mitenyi Biotec	AF12-7H3
CD16 APC	Mitenyi Biotec	REA423
CD158a/h (KIR2DL1/DS1) APC	Mitenyi Biotec	REA1010
CD158b (KIR2DL2/DL3) PE	Mitenyi Biotec	REA1006
CD158i (KIR2DS4) APC	Mitenyi Biotec	REA860
CD158e1/e2 (KIR3DL1/S1) APC	Mitenyi Biotec	REA168
CD158f (KIR2DL5) PE	Mitenyi Biotec	REA955
CD158e/k (KIR3DL1/DL2) APC	Mitenyi Biotec	REA970
NKG2A (CD159a) APC	Mitenyi Biotec	REA110
CD45 APC	Mitenyi Biotec	REA747
REA Control Antibody (S), human IgG1, PE	Mitenyi Biotec	REA293
REA Control Antibody (S), human IgG1, APC	Mitenyi Biotec	REA293
Isotype Control Antibody, mouse IgG2a, PE	Mitenyi Biotec	S43.10
Isotype Control Antibody, mouse IgG1, APC	Mitenyi Biotec	IS11-12E4.23.20
CD94 PE	BD biosciences	HP-3D9
NKP44 (CD336) PE	BD biosciences	P44–8
NKP46 (CD335) APC	BD biosciences	9E2/NKp46
FasL (CD178) APC	BD biosciences	NOK-1
Mouse IgG1 κ isotype control APC	BD biosciences	MOPC21

2.5 Functional Assays

1. Victor^3 plate reader (PerkinElmer, Waltham, MA).
2. DELFIA EuTDA Cytotoxicity Reagents kit (PerkinElmer).
3. APC-conjugated CD107a antibody (BD Biosciences).
4. GolgiStop™ containing monensin (BD Biosciences).
5. U-bottom 96-well plate.
6. Anti-CD56 PE antibody staining (Miltenyi Biotec) (*see* **Note 1**).

3 Methods

3.1 Peripheral Blood Mononuclear Cells and Primary Natural Killing Cell Isolation

1. Isolate fresh human PBMCs from the healthy donors' leukocyte reduction system cones with Ficoll-Paque™ Plus through density gradient centrifugation.
2. Proceed directly with the isolated PBMCs to the next step or freeze them down with freezing media (AB serum +10% DMSO) in liquid nitrogen (*see* **Note 2**).
3. Apply 3–4 × 10^7 of PBMCs for the NK cell isolation according to the manufacturer's instructions (Miltenyi Biotec). NK cells are negatively selected by the magnetic beads.
4. Determine the purity of NK cells after isolation by flow cytometry. (Fig. 2a; *see* **Note 3**).

3.2 NK Cell Activation and Expansion

1. Co-culture 5 × 10^6 NK cells with 100 Gy gamma-irradiated K562 aAPCs (with membrane-bound IL-15, IL-21, and 41BBL) at a 1:1 ratio in 10 mL AIM-V supplemented with 5% AB serum in a T75 Nunc EasyFlask (place upright in a 37 °C, 5% CO_2 incubator), with 2.5 μg/mL of DNase and 50 IU/mL of IL-2 added for 7 days.
2. Change half volume of the media every 2–3 days and replenish IL-2 according to the full volume concentration until day 7. Cell number should increase by five- to tenfold at the end of the culture.

3.3 Generation of CAR-NK Cells with piggyBac *Transposon System*

1. Harvest the NK cells and wash them three times with Opti-MEM buffer (*see* **Note 4**).
2. Resuspend the cell pellet in P3 Primary Cell Nucleofector ™ Solution at a density of 5 × 10^6 cells per 90 μL of the solution.
3. Prepare the completed media (AIM-V with 2.5 μg/mL of DNase and 50 IU/mL of IL-2).
4. The preparation of the *piggyBac* transposon platform plasmid has been described previously [11, 16]. Add 5 μg of *piggyBac* transposase plasmid and 10 μg NKG2D CAR plasmid (1:2 ratio) into the cell suspension.

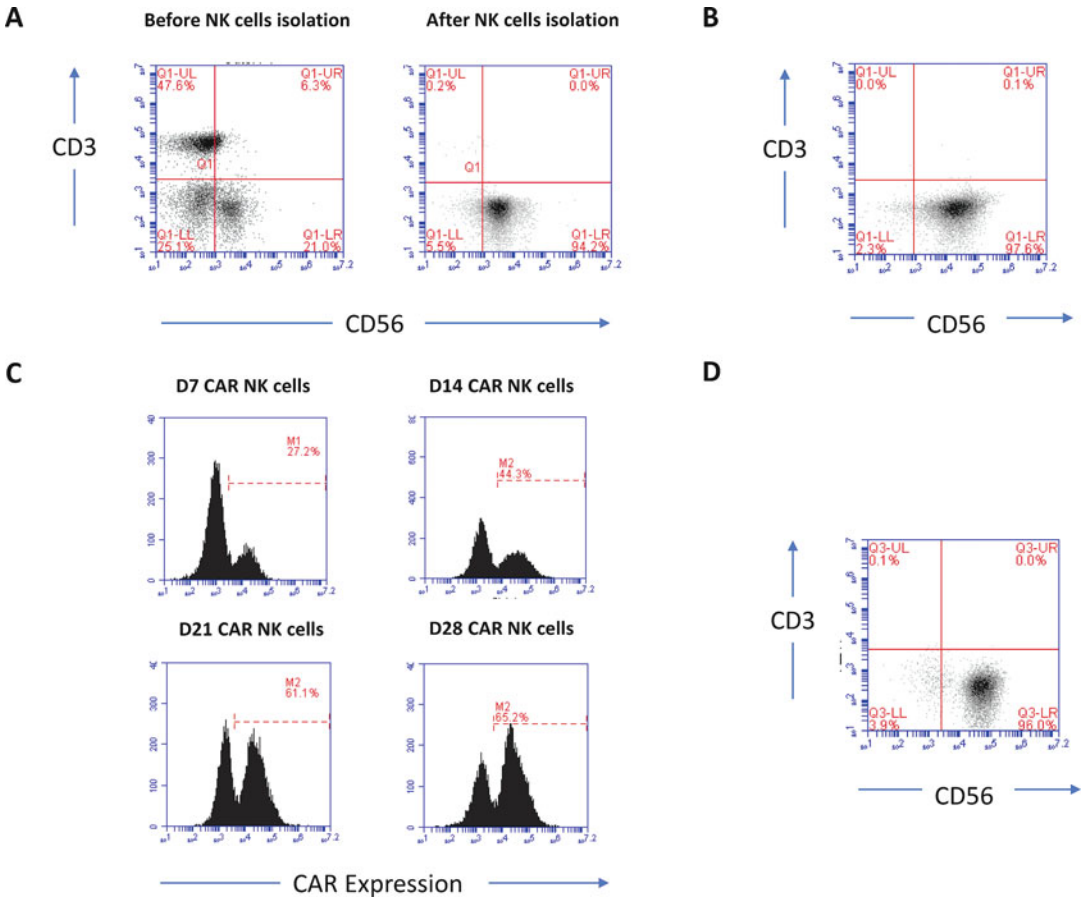

Fig. 2 Flow cytometry analysis of NK cells and CAR-NK cells through the manufacturing process. (**a**) A representative panel of flow cytometry plots to show NK cells purity before and after NK cells isolation. (**b**) A representative flow cytometry plot to show NK cells purity after seven days expansion with K562 aAPCs. (**c**) A representative panel of flow cytometry plots to show the antigen-specific enrichment of NKG2D CAR-NK cells stimulated by K562 aAPCs. The CAR-NK cells are gated base on CD56+ cells and the CAR expression is detected by the Streptavidin II tag (STII). (**d**) A representative flow cytometry plot to show final CAR NK cells purity

5. Transfer the cells and DNA mixture to a Nucleocuvette™ Vessel (*see* **Note 5**) and electroporate with the 4D-Nucleofector™ system with the pre-set program EN-138.
6. Slowly add 500 μL of the completed media (refer to **step 3**) into the Nucleocuvette™ Vessel and place it in a 37 °C, 5% CO_2 incubator for 5–10 min.
7. Resuspend and transfer the cell suspension to a new T75 Nunc EasYFlask with the dropper provided by the manufacturer.
8. Add 9.5 mL of completed media to the culturing flask.

3.4 Large-Scale CAR-NK Cell Enrichment and Expansion

1. Enrich and expand the CAR-NK cells with 100 Gy gamma-irradiated K562 aAPCs at an E:T ratio of 1:1 for four rounds, with 7 days per round (*see* **Note 6**).
2. Replenish the K562 cells at a 1:1 ratio every 7 days with the completed media (refer to **step 3**).
3. Change half of the media and replenish IL-2 (50 IU/mL) for full volume every 2–3 days.
4. Harvest the CAR-NK cells at the end of the fourth round. The number of NK cells should reach around 8000 folds.

3.5 Phenotyping of CAR-NK Cells

1. Examine the presence of NKG2D CAR on NK cells by the THE™ NWSHPQFEK Tag antibody, due to the specially designed plasmid construction with the expression of a streptavidin tag (STII tag) (*see* **Note 7**). The CAR expression should generally reach 50% or higher for the final CAR-NK cell product (*see* **Note 8**).
2. Determine the purity of NK cells by CD3 and CD56 expression, similar to the previous steps (Fig. 2d).
3. Test for other typical phenotypic markers of NK cells by flow cytometry analysis: CD16, Nkp44, Nkp46, CD94, FasL, TRAIL, NKG2A, KIRs (CD158b, CD158e/k, CD158a/h, CD158e1/e2, CD158i). The phenotype of CAR-NK cells generated by this protocol should not be affected compared to the nongenetically modified NK cells [15].

3.6 Functional Characterization of CAR-NK Cells by In Vitro Cytotoxicity Assay (See Note 9)

Cytotoxicity assay should generally follow the manufacturer's protocol (three-hour Europium-release assay with DELFIA EuTDA Cytotoxicity Reagents kit).

1. Resuspend target cells (1×10^6 cells) in 1 mL cell culture medium (AIM-V + 5% AB serum) and label them with 2 μL of bis(acetoxymethyl)2,2′:6′,2″-terpyridine-6,6″-dicarboxylate (BATDA) in a 37 °C, 5% CO_2 incubator for 20 min with two times washing by PBS subsequently (*see* **Note 10**).
2. Fully suspend the target cells in the cell culture medium at the concentration of 5 x 10^4 cells/mL.
3. Mix the CAR-NK cells with the target cells in triplicates at different effector/target (E:T) ratios as required and seed into 96-well U-bottom plates with 200 μL of culture medium (5×10^3 target cells each well).
4. Prepare the spontaneous release of target cells by seeding the target cells alone in triplicates into 96-well U-bottom plates.
5. Prepare the maximal release of labelled target cells by adding 10 μL DEILFA lysis buffer into the 190 μL culture medium with the target cells in triplicates into 96-well U-bottom plates.

6. After 3 h of incubation in a 37 °C, 5% CO_2 incubator, centrifuge the culture plate at 400 g for 5 min and transfer 20 μL of the supernatant into a flat-bottom reading plate with 200 μL DELFIA Europium solution added.
7. Shake the plate for 15 min with an orbital shaker at the speed of 250 rpm and then read values with the Victor^3 plate reader (*see* **Note 11**).

3.7 Functional Characterization of CAR-NK Cells by CD107a Degranulation Assay

1. Wash CAR-NK cells with 1× PBS once and resuspend them into 1 mL AIM-V supplemented with 5% AB serum.
2. Add APC-conjugated CD107a antibody and GolgiStop™ according to the manufacturer's protocol into the CAR-NK cell suspension.
3. Prepare target cells by washing them with 1× PBS, resuspending into AIM-V supplemented with 5% AB serum and seeding into a U-bottom 96-well plate at the concentration of 2×10^5 cells/100 μL/well (*see* **Note 10**).
4. Seed CAR-NK cells at a 1:1 E:T ratio (ratio could be amended based on experiment settings) with target cells in a 37 °C, 5% CO_2 incubator for 5 h.
5. After incubation, stain the cells with anti-CD56 PE antibody according to the manufacturer's protocol.
6. Proceed to flow cytometry analysis for detecting CD56 and CD107a expression.

4 Notes

1. In vivo assays are not discussed in this protocol due to their high inconsistency between laboratories. Researchers can consider NSG mice with xenograft as requested.
2. It is recommended to keep the frozen cells at an amount of 5×10^7 per vial. Thaw one vial of PBMC stock with warm culture medium (AIM-V with 5% AB serum) and rest them overnight in a 37°C, 5% CO_2 incubator with 20–30 mL of fresh culture media supplemented with DNase (2.5 μg/mL) in a T75 Nunc EasYFlask.
3. After NK cell isolation, the purity of NK cells (CD3-CD56+) should reach about 90%. The T cell percentage (CD3+) should be generally less than 1%.
4. The purity of NK cells (CD3-CD56+) and T cell percentage (CD3+, <1%) should be confirmed by flow cytometry again in case of T cell contamination before proceeding to electroporation (Fig. 2b).

5. Avoid bubbling in the solution by carefully pipetting and gently tapping the side of the cuvette 2–3 times.
6. The gamma-irradiated K562 aAPCs naturally express two of the eight NKG2D ligands (NKG2DLs) to achieve the NKG2D CAR-NK cell antigen-specific enrichment and expansion [15]. For other CAR construct enrichment and expansion, specific aAPCs can be designed and prepared accordingly.
7. The examination of CAR expression should be carefully designed. Some scFv regions may have commercial antibodies available for detection. Otherwise, unique markers (e.g., STII tag) should be constructed into the CAR plasmid for co-expression.
8. CAR expression can also be detected after each round of co-culturing with K562 aAPCs to monitor the CAR-NK cell enrichment and expansion (D7-D28, Fig. 2c).
9. Functional assays are for recommendations. Other assays such as ELISA, ELISPOTS, or intracellular cytokine staining can also be considered.
10. The target cells can vary depending on the CAR constructs used.
11. The killing efficacy should be calculated by applying the following formula:

$$\text{Specific Lysis} = \frac{\text{Experimental release (counts)} - \text{Spontaneous release (counts)}}{\text{Maximal release (counts)} - \text{Spontaneous release (counts)}} \times 100\%$$

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Chapter 19

Engineering Probiotic *E. coli* Nissle 1917 for Release of Therapeutic Nanobodies

Candice Gurbatri and Tal Danino

Abstract

Bioengineered probiotics enable new opportunities to improve cancer treatment strategies due to their tumor-colonizing capabilities. Here, we will describe the development of a probiotic *E. coli* Nissle 1917 platform encoding a synchronized lysis mechanism for the localized and sustained release of blocking nanobodies against immune checkpoint molecules like programmed cell death protein-ligand 1 and cytotoxic T lymphocyte–associated protein-4. Specifically, we will detail the experimental protocols needed to (1) encode and validate binding of recombinantly produced checkpoint blockade nanobodies, (2) evaluate the therapeutic efficacy and safety of the probiotic platform in syngeneic tumor-bearing mice, and (3) analyze the immunophenotype of the tumor microenvironment.

Key words Bacteria, Probiotic, Cancer therapy, Checkpoint inhibitors, Immunotherapy, Nanobody

1 Introduction

In recent years, cancer immunotherapy has demonstrated unparalleled successes with immunotherapies, including antibodies against immune checkpoints like programmed cell death protein-ligand 1 (PD-L1) and cytotoxic T lymphocyte–associated protein-4 (CTLA-4), which have significantly improved clinical outcomes for multiple cancer types [1, 2]. Despite overall efficacy, systemic delivery of immunomodulatory antibodies produce a range of unpredictable toxicities [3–7]. Furthermore, a combination of immunotherapies exhibits additive autoimmune toxicity [8, 9] and can lead to favoring of less efficacious monotherapies or eventual drug discontinuation [10, 11].

In parallel to these immunological findings is the rapid rise of genetic engineering technologies, enabling the repurposing of bacteria as safe and selective cancer therapies [12, 13]. Due to their preferential colonization of tumors, bacteria are a natural chassis for the localized delivery, with multiple studies examining the release of

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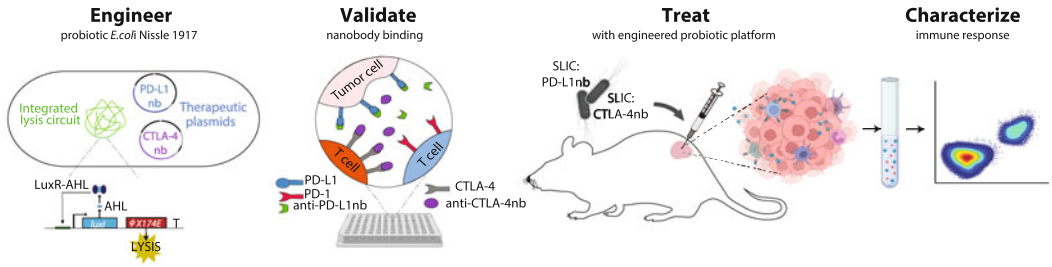

Fig. 1 Schematic of the protocol: (**a**) engineer SLIC strains to encode blocking nanobodies against PD-L1 and CTLA-4 targets, (**b**) validate recombinantly produced nanobody binding, (**c**) treat subcutaneous syngeneic lymphoma tumors in immunocompetent mice via intratumoral injections, and (**d**) interrogate the immunophenotype of excised tumors after bacteria treatment using flow cytometry methods

proteins not inherent to bacteria including cytokines [14–21], antigens [22–25], and, more recently, of single-chain variable fragments, or nanobodies [26–29]. Additionally, they can be further engineered with active release mechanisms to maximize intratumoral therapeutic accumulation [30, 31].

Here, we will describe the use of probiotic *E. coli* Nissle 1917 (EcN) that naturally homes to the tumor core. Once localized, they were engineered to recombinantly produce blocking nanobodies against established immune checkpoint molecules and actively lyse once a critical density is reached, thereby releasing the immunotherapeutics. A small fraction of bacteria remain to reseed the population and the cyclical delivery continues [30]. We subsequently integrated this lysis circuit into the genome of EcN (SLIC). Protocols needed to engineer probiotic strains, validate nanobody binding, evaluate therapeutic efficacy in murine models, and characterize the immune response are provided (Fig. 1). Using these experimental designs, we have previously demonstrated that a single injection of this engineered probiotic outperforms analogous clinically relevant antibodies, resulting in the delay or complete regression of multiple tumor types in immunocompetent mice [32].

2 Materials

2.1 Cell Lines (See Notes 1 and 2)

1. A20 (ATCC, TIB-208).

2.2 Plasmids

1. p246-axe/txe stabilized plasmid was modified to remove the *luxCDABE* cassette (from reference [33]).
2. PD-L1 (pCG01 therapeutic plasmid from reference [32]).
3. CTLA-4 (pCG01 therapeutic plasmid from reference [32]).

2.3 Bacterial Strains

1. *E. coli* Nissle 1917 encoding a genomically integrated *lux-CDABE* cassette (EcN-lux) was obtained from Dr. Sangeeta Bhatia. Full details for this bacteria can be found in reference [34].
2. SLIC strain encoding a genomically integrated synchronized lysis operon will be described in the *Methods* and more details about this variant and the original lysis variants can be found in references [30, 32], respectively.
3. SLIC:PD-L1nb: lysing EcN-lux strain expressing PD-L1nb [32].
4. SLIC:CLTA-4nb: lysing EcN-lux strain expressing CTLA-4nb [32].
5. NiCo21(DE3) *E. coli* (NEB, C2529) for downstream protein purification.

2.4 Mice (See Notes 3–6)

1. Female BALB/c mice (4–6 weeks old) were purchased from Taconic Biosciences.

2.5 General Reagents

1. RPMI 1640 (Gibco, Thermo Fisher, 11,835,030).
2. Fetal bovine serum (FBS) (Gibco, Thermo Fisher, 10-437-028).
3. Penicillin–streptomycin (CellGro, Thermo Fisher, 15-070-063).
4. LB broth (Lennox, Thermo Fisher, BP9722-500).
5. Kanamycin (Sigma-Aldrich, 10,106,801,001).
6. Ampicillin (Sigma-Aldrich, A9518).
7. Trypan blue (Gibco, Thermo Fisher, 15,250,061).
8. LB agar (Lennox, Thermo Fisher, 22,700,025).
9. 1× DPBS (Thermo Fisher, 14,190,144).
10. 190 proof ethanol (Thermo Fisher, 22-032-600).
11. N-(β-Ketocaproyl)-L-homoserine lactone (AHL) (Sigma, K3007).
12. 1-Thio-D-galactopyranoside (IPTG) (Thermo Fisher, 15,529,019).
13. 2-Mercaptoethanol (Sigma, M6250).
14. Gibson Assembly Master Mix (NEB, NC0349689).
15. QIAgen Spin Miniprep Kit (Qiagen, 27,104).

2.6 Equipment for Tissue Culture

1. T25 tissue culture flask (Falcon, 10-126-9).
2. 6-well microplate (Thermo Fisher, 08-772-1B).
3. Serological Stripettes (Falcon, 13-675-49).
4. Serological pipet gun (Rainin, 17,011,733).

5. Pipet (Rainin, 30,456,871).
6. Pipet tips (Rainin, 30,389,225).
7. Cell counter slides (Thermo Fisher, C10228).
8. 15-mL conical tubes (Falcon, Thermo Fisher, 14-959-70C).
9. 37 °C CO_2 incubator (Thermo Fisher Scientific Forma Steri-Cycle Model 370).
10. 37 °C water bath (Thermo Fisher Scientific, TSGP02).
11. Centrifuge (Eppendorf 5804R, 022628146).
12. Cell counter (Invitrogen, Thermo Fisher, AMQAX2000).
13. Vacuum line.

2.7 Equipment for Bacteria Culture and Transformation

1. 12-mL culture tubes (Falcon, Thermo Fisher, 14-959-11B).
2. 37 °C incubator shaker (Thermo Fisher, SHK6000).
3. NanoDrop One[c] (Thermo Fisher, ND-ONEC-W).
4. Plastic cuvettes (Fisher/BrandTech, 14-377-017).
5. 37 °C CO_2 incubator (Thermo Fisher Scientific Forma Steri-Cycle Model 370).
6. Petri dishes (VWR, 25384-094).
7. Model 505 Sonic Dimembrator (Thermo Fisher, FB505) with a 1/8 inch diameter probe.
8. Cryovials (VWR, 66008-728).
9. SOC media (Thermo Fisher, 15-544-034).
10. Gene Pulser/MicroPulser electroporation cuvettes, 0.1-cm gap (Bio-Rad, 1,652,089).
11. MicroPulser Electroporator (Bio-Rad).

2.8 Equipment for Mouse Experiments

1. Syringe 1 mL (Thermo Fisher Scientific, 14-841-32).
2. Syringe 5 mL (Thermo Fisher Scientific, 14-955-458).
3. Insulin syringe – 29G (Thermo Fisher Scientific, 14-841-32).
4. Isoflurane isothesia solution (Henry Schein, 029405).
5. Compressed oxygen (Airgas).
6. Anesthesia machine (Avante, 10,150).
7. Caliper (Thermo Fisher Scientific, 14-648-17).
8. Mouse diet (PicoLab® Rodent Diet 20, 5053).
9. Animal cages – 5 mice per cage according to IACUC protocol.
10. IVIS Lumina LT Series III In Vivo Imaging (PerkinElmer, CLS136331).
11. Benchtop weighing scale.

2.9 Reagents for Ex Vivo Analysis and Flow Cytometry

1. Corning cell strainer, 100 micron (Thermo Fisher, 431,752).
2. 0.22-micron sterile syringe filter (Thermo Fisher, 09-720-511).
3. 96-well microplate V bottom (Thermo Fisher, 07-200-109).
4. ACK lysing buffer (Thermo Fisher, A1049201).
5. 5-mL microcentrifuge tube (Thermo Fisher, 03-391-276).
6. BD LSRFortessa cell analyzer (BD Biosciences).

2.10 Reagents for Protein Visualization and Purification

1. Pierce Coomassie Plus (Bradford) Assay Kit (Thermo Fisher, 23,236).
2. HisPur Ni-NTA Resin (Thermo Fisher Scientific, PI88222).
3. MES SDS Running Buffer (Thermo Fisher, B0002).

2.11 Imaging and Software

1. Living Image.
2. FlowJo Version 10.

3 Methods

3.1 Plasmids and Strain Engineering

3.1.1 Therapeutic Plasmids

1. Use Gibson assembly methods to construct a plasmid whereby a geneblock (IDT) encoding a *tac* promoter and the *E. coli* codon optimized for either (a) a PD-L1 nanobody (PD-L1nb, RCSB PDB: 5DXW) or (b) CTLA-4 (CTLA-4nb, RCSB PDB: 5E03) with a C-terminus HA tag is assembled to a linearized p246-axe/txe stabilized plasmid (*see Materials: Plasmids*) (*see* **Note 7**).

3.1.2 SLIC Strain

1. Use Gibson assembly methods to clone a geneblock (IDT) encoding a constitutively produced *luxR* and a single operon where *luxI a*nd *ϕX174E* are driven by the *luxI* promoter onto the PAH162 CRIM plasmid [35]. Follow the established CRIM protocol [35] to integrate this plasmid into the *ϕ80* site of EcN-lux (*see* **Notes 8–11**).

3.1.3 E. coli Expression Strains for Downstream Purification

1. Use Gibson assembly methods to construct a plasmid containing a geneblock (IDT) encoding an *E. coli*-optimized sequence for either the PD-L1nb or the CTLA-4nb with a C-terminus 6xHis tag into the multiple cloning site of a 1-thio-D-galactopyranoside (IPTG)-inducible pET vector containing ampicillin resistance (100 ug/mL).

3.1.4 Electrocompetent EcN-lux Strains (See **Note 12**)

1. Make an overnight culture of EcN-lux in LB media.
2. The next day, subculture overnight 1:100 into fresh LB media.
3. Incubate in a shaking 30 °C incubator.

4. Check the OD_{600} every hour and remove when the culture reaches an OD_{600} of ~0.4–0.5 (log phase).
5. Incubate culture on ice for 10 min to stop bacteria cell growth.
6. Spin at 3000 r.c.f. in a refrigerated centrifuge for 10 min.
7. Decant supernatant and wash in 50 mL DSPBS.
8. Repeat **step 6**, three times.
9. After the last wash, decant supernatant and resuspend in 200 μl of 15% glycerol.
10. Aliquot 50–100 μl into pre-chilled Eppendorf tubes.
11. Store at −80 °C until cells are ready for electroporation.

3.2 Tissue Culture

3.2.1 Thaw Frozen Tumor Cells from a Liquid Nitrogen Stock

1. Prepare tumor cell culture medium: A20 cells are cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.01% 2-mercaptoethanol.
2. Warm up tumor cell culture medium to 37 °C in water bath.
3. Sterilize biosafety cabinet using UV light and wipe down all working surfaces with 70% ethanol. To ensure a sterile hood, spray ethanol onto any bottles or materials entering the biosafety cabinet.
4. Pipette 8 mL of tumor cell culture media into a 15-mL conical tube.
5. Thaw a cryovial of cancer cells by placing it into a 37 °C water bath. This process should take 45–60 s.
6. Take out the cryovial from the water bath when all but a small iceberg remains in the vial and transfer to the biosafety cabinet.
7. Add 1 mL of tumor cell culture medium to the cryovial and gently resuspend the tumor cells using a 1-mL pipette.
8. Add 1 mL of warmed tissue culture media to the cryovial dropwise and transfer cryovial contents gently into the conical with tissue culture media already inside, bringing its total volume to 10 mL.
9. Centrifuge at 150 r.c.f. for 5 min.
10. Aspirate the growth medium using vacuum suction inside the biosafety cabinet.
11. Gently resuspend the tumor cells in 1 mL of warm tumor cell culture medium.
12. Take out 10 μl tumor cells and mix with 10 μl trypan blue staining (1:1 ratio). Transfer 10 μl of the mixture to cell counting chamber slides to calculate the concentration of cells.
13. Add 1×10^6 tumor cells to 5 mL of tumor cell culture medium in a T25 flask.

14. Transfer the medium containing tumor cells into the culture flask. Gently shake to evenly distribute the tumor cells in flask.
15. Place the culture flask into the 37 °C tissue culture incubator maintained at 5% CO_2.
16. After 2–3 days of culture, observe the tumor cells under the brightfield microscope. The cells are ready to be expanded when they cover ~80% of the flask's bottom surface.

3.2.2 Expanding Tumor Cell Lines

2. Warm up tumor cell growth medium to room temperature using the 37 °C in a water bath.
3. Remove the tissue culture flask from the incubator and transfer to the biosafety cabinet.
4. Pipette the media in the flask into a 15- or 50-mL conical tube. Since A20 cells are not adherent, these cells do not need to be trypsinized.
5. Centrifuge the tube at 150 r.c.f. for 5 min at room temperature.
6. Count cells using **step 12** above and determine if there are enough cells for downstream experiment by expanding into larger tissue culture flasks.

3.3 Characterization of Nanobodies

3.3.1 Purification of Nanobodies

1. Transform the pET vectors encoding either the PD-L1 or CTLA-4 nanobody into chemically competent NiCo21(DE3) *E. coli*. Select for the transformants with ampicillin resistance (100 μg/mL) and incubate agar plates at 37 °C overnight.
2. Choose a single colony and grow at 37°C until an OD_{600} of ~0.9 is reached.
3. Induce with 1 mM IPTG for 16 h at 30°C.
4. Centrifuge cells for 10 min at 4000 r.c.f.
5. Resuspend in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), 15 mL per L of culture.
6. Add 100 μl protease inhibitor cocktail (Sigma P8849) per L of culture.
7. Lyse cells by sonicating using the following cycle: 60% amplitude, 4 s *on*, 2 s *off* for 5–10 min per cycle and complete 3 cycles total (*see* **Note 13**).
8. Centrifuge lysates for 30 min at 10,000 r.c.f. in a refrigerated centrifuge (temperature controlled to 4°C) to remove cellular debris.
9. Purify soluble protein using a Ni-NTA (Qiagen) affinity chromatography per the manufacturer's instructions with the following modifications: increase imidazole concentration in wash buffer to 35 mM imidazole and elute in 250 mM imidazole for

CTLA-4nb collection or 100 mM imidazole for PD-L1nb collection.

10. Analyze the flow-through, wash, and elution fractions on SDS-PAGE to confirm the protein size is correct and is in the elution fraction.
11. Dialyze purified proteins in DPBS using a cellulose dialysis membrane with a 3.5-kDa molecular weight cutoff.
12. Filter dialyzed protein through a 0.22-micron filter. Aliquots can be stored at 4 °C until needed.
13. Use a Bradford Colorimetric Assay and its associated protocol to quantify purified protein.

*3.3.2 Collect Probiotically Produced PD-L1 and CTLA-4 Protein (*See ***Notes 14****)*

1. Use electroporation methods to transform each PD-L1 and CTLA-4-expressing plasmid into separate EcN-lux electro-competent strains.
2. Select for the transformant on LB agar plates supplemented with 50 ug/mL kanamycin. Pick a single colony and grow in LB media supplemented with 50 μg/mL kanamycin at 37°C overnight.
3. Subculture overnight into 1:100 of 50 mL of fresh LB media supplemented with 50 μg/mL kanamycin.
4. Centrifuge culture for 5 min at 3000 r.c.f.
5. Decant the supernatant and resuspend the bacterial pellet in 5 mL of 1640 RPMI media (*see* **Note 15**).
6. Freeze the samples at −80°C and thaw in a 30°C incubator.
7. Repeat **step 6**, five times.
8. After the final thaw step, centrifuge the sample for 5 min at 3000 r.c.f. to remove bacterial debris.
9. Filter 1 mL of the resulting lysate through a 0.22-micron syringe filter.
10. Confirm and visualize protein of interest in the lysate using western blot technique (*see* **Note 16**).

*3.3.3 PD-L1 Nanobody (PD-L1nb) Binding (*See ***Note 17****)*

1. In a tissue culture hood, seed 1×10^7/mL A20 cells in a 96-well V-bottom plate with a working volume of 100 μl (*see* **Note 18**).
2. Centrifuge cells at 450 r.c.f. and resuspend in Fc Block (Tonbo, 1:1000) prepared in FACS buffer (1% BSA, 2.5 mM EDTA in DPBS).
3. Incubate for 10 min at 4°C.
4. During the above incubation, prepare ten-fold dilutions of lysate samples collected in "*Characterization of Nanobodies*" (*see* **Note 19**).

5. Remove the well plate from 4°C and add 50 μL of the lysates to the cells or purified PD-L1nb (rPD-L1nb, 0–10 ug) to the wells.
6. Add fluorophore conjugated anti-PD-L1 antibodies (MIH7 clone from Biolegend or 10F.9G2 clone from Biolegend) to the wells at the manufacturer's recommended concentrations (*see* **Note 20**).
7. Incubate for 2 h at room temperature.
8. Wash three times in ice-cold DPBS.
9. Analyze on a BD LSRFortessa cell analyzer (*see* **Note 21**).

3.3.4 CTLA-4 Nanobody Binding

1. Harvest a spleen from a mouse (*see* **Note 22**).
2. In the tissue culture hood, place a 100-micron filter on a 50-mL conical.
3. Place spleen on the filter and pour 1 mL of 1640 RPMI media on the filter (*see* **Note 23**).
4. Mash the spleen with the rough end of a plunger taken from a 5-mL syringe, with the goal of breaking up the spleen into smaller parts.
5. Add 10 mL of RPMI media and squirt onto the filter, to further break apart the spleen.
6. Add 10 mL of media.
7. Spin the conical at 2500 r.c.f. for 5 min at room temperature and decant the supernatant.
8. Add 2 mL of ACK lysis buffer and allow the reaction to proceed for 2 min.
9. Decant the supernatant.
10. Resuspend in 5 mL RPMI media and count the number of cells using a cell counter.
11. In a 6-well tissue culture plate, seed 2×10^6 splenocytes per ml.
12. Stimulate splenocytes with PMA (50 ng/mL; Sigma-Aldrich) and ionomycin (1 nM; Calbiochem) and incubate at 37 °C for 48 h. Make sure to keep one well unstimulated to serve as a negative control.
13. Transfer 100 μl from each well into a 96-well V-bottom plate.
14. Incubate unstimulated and stimulated splenocytes with purified CTLA-4nb for 2 h.
15. Wash three times with ice-cold PBS.
16. Detect the extracellular binding with an anti-histidine (Pena-His Alexa Fluor 647 Conjugate, Qiagen) (*see* **Note 24**).
17. Analyze samples on a BD LSRFortessa cell analyzer.

3.4 Therapeutic Evaluation in Mouse Models

3.4.1 Establishment of Subcutaneous Model

1. Obtain BALB/c mice and acclimate them in the facility for a week before tumor inoculations (*see* **Note 25**).
2. Culture tumor cells described in the *Tissue Culture: Expanding tumor cell line* subsection to a density of 5×10^7/mL and resuspend in ice-cold DPBS (*see* **Note 26**).
3. Anesthetize animals following appropriate IACUC protocol.
4. Once mice are asleep, use forceps to create a "tent" by lifting the skin on the hind flank of the mouse. Using a 29-gauge insulin syringe, inject the cells slowly being careful to not pierce through the other side of the "tent."
5. Release forceps before slowly pulling out the needle. A bubble should transiently form indicating that the injection was indeed in the subcutaneous space.
6. Monitor mice, and after the mice wake up and start moving inside the cage, place the cage in the appropriate place in the animal facility. Tumors will be visible and palpable over the next 14–20 days.
7. Once tumors are palpable, measure and record tumor volume of tumor-bearing BALB/c mice using calipers twice a week. CT26 tumor volumes are calculated by measuring the length, width, and height of each tumor ($V = L \times W \times H$). Since A20 tumors are less solid, measurement of tumor z dimension is highly variable, and therefore, total volume should be calculated as length $\times$ width^2 $\times$ 0.5 [36]. Tumors are ready for treatment when they grow to approximately 150–200 mM^3.
8. Randomize the mice so that the control and treatment groups have comparable tumor volumes. For statistical purposes, there should be ~5 mice per group and all groups should have comparable sample sizes.

3.4.2 Therapeutic Bacteria Preparation

1. Use electroporation methods to transform each PD-L1 and CTLA-4-expressing plasmid into separate SLIC electrocompetent strains.
2. Select for the transformant on LB agar plates supplemented with 50 μg/mL kanamycin and 0.2% glucose. Pick a single colony and grow in LB media supplemented with 50 μg/mL kanamycin and 0.2% glucose at 37 °C overnight.
3. Make glycerol stocks for future use by mixing a 1:1 ratio of the overnight culture with 50% glycerol into a cryovial. Cryovials can be stored at −80 °C.
4. The night before the start of treatment, grow the SLIC:PD-L1nb and SLIC:CTLA-4nb strains overnight in LB media supplemented with 50 μg/mL kanamycin and 0.2% glucose (*see* **Note 27**).
5. Next morning, subculture overnight into 1:100 50 mL of fresh LB media supplemented with 50 μg/mL kanamycin and 0.2% glucose.

6. Grow the culture in the 37 °C incubator shaker until the OD_{600} reaches approximately 0.1–0.3 (log growth phase).
7. Place the bacterial culture on ice for 10 min to halt growth.
8. Centrifuge the bacteria culture at 3000 r.c.f for 10 min in 4 °C and discard the supernatant.
9. Resuspend the pellet with sterile 50 mL 1× DPBS by gently pipetting up and down (*see* **Note 28**).
10. Wash bacteria by repeating **steps 5** and **6**, three times.
11. Adjust the OD_{600} of each therapeutic strain to 0.5 (~5×10^7 CFU/mL).
12. Make an equal parts cocktail of SLIC:PD-L1nb and SLIC-CTLA-4nb, where the OD_{600} of the combined cocktail is 0.5.

3.4.3 Therapeutic Bacteria Administration

1. Load the bacteria into the insulin syringe with a 29-gauge needle.
2. Insert the needle in the tumor at a 45-degree angle, aiming for the central area of the tumor. Pull out slightly and then very slowly inject a total volume of 30–50 μl per tumor (*see* **Notes 29** and **30**).
3. Measure tumor volume as in the *Establishment of subcutaneous model* subsection **step 7** and body weight using a benchtop scale every 2–3 days.

3.4.4 Monitoring In Vivo Bacteria Dynamics

1. All bacterial strains used were luminescent (integrated *luxCDABE* cassette) so they could be visualized with the in vivo imaging system (IVIS) [34]. To track lysing bacterial populations, take IVIS images every 10–12 h. Living Image software can be used to quantify luminescence.

3.5 Evaluation of Safety

3.5.1 Biodistribution

1. At the study endpoint or when the tumor reaches the sacrifice limit as delineated in the IACUC protocol, assess bacterial localization by excising and weighing tumors, spleen, and liver.
2. Place tissues aseptically into 5 mL of DPBS in gentleMACS cell dissociator C tubes, one tissue per tube, and homogenize using program E.
3. Serially dilute homogenates 1:100 six times in DPBS and spot 5 μl of each dilution onto an LB agar plate with erythromycin selection at 50 μg/mL. To another set of plates, addition of 50 μg/mL kanamycin can assist in calculating the colonies that have retained the therapeutic plasmid (*see* **Note 31**).
4. Incubate plates overnight at 37 °C.
5. Calculate colony-forming units (CFUs) for each sample normalized to weight of tissue to generate CFU/g tissue.

3.5.2 Characterization of Immune Response

1. Between 6 and 8 days after treatment, aseptically excise the tumor, and weigh 200 mg of tumor tissue per tube (*see* **Note 32**).
2. Place tumor tissue in a 5-mL microcentrifuge tube with 3 mL of isolation buffer (RPMI 1640 supplemented with 5% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 10 mM Hepes).
3. Mechanically homogenize by using scissors to manually chop the tumor into very fine pieces.
4. Add collagenase A (1 mg/mL, Roche) and DNAse I (0.5 ug/mL) to the tube to assist with digestion.
5. Incubate tube for 1 h in 37 °C at 150 rpm.
6. Filter cells through a 100-micron cell strainer, mash through using a syringe plunger, and wash with isolation buffer until all tissue is through the filter.
7. Bring volume to 20 mL with isolation buffer, and spin at 450 r.c.f. for 5 min at 4 °C.
8. Resuspend in 1 mL ice-cold PBS.
9. Transfer 100 μL per well to a 96-well V-bottom plate, top up to 200 μL with ice-cold PBS, and spin at 450 r.c.f. for 5 min at 4C.
10. Resuspend in 200 μL of mixture to measure cytokine production by T cells by stimulating lymphocytes for 3 h with PMA (50 ng/ml; Sigma Aldrich) and ionomycin (1 nM; Calbiochem) in the presence of brefeldin A in isolation buffer.
11. Spin down at 450 r.c.f. for 5 min at 4 °C.
12. Decant supernatant from plate, exclude dead cells by resuspending cells with 100 μL viability dye (Ghost Dye Red 780, 1:1000 in PBS, Tonbo Biosciences), and incubate 15 min on ice.
13. Add 100 μL FACS buffer to each well, spin for 5 min at 450 r.c.f. at 4 °C, and decant supernatant.
14. Resuspend in 50 μL 2× Fc Block (1:500 in FACS Buffer), and incubate for 10 min at 4 °C.
15. Prepare extracellular antibody cocktail for staining. Some possibilities include anti-B220 (BD), anti-CD4 (Tonbo), anti-CD8 (eBioscience), and anti-NKp46(BD).
16. Add 50 μL 2× extracellular antibody cocktail to each well and incubate for 30 min at 4C.
17. Add 100 μL FACS buffer to each well, spin for 5 min at 450 r.c.f, and decant supernatant.
18. Resuspend in 200 μL FACS buffer, spin for 5 min at 450 r.c.f., and decant supernatant.

19. Fix cells using eBio fix/perm buffer from the Foxp3 Transcription Factor Staining Buffer Kit (Tonbo Biosciences) per manufacturer's protocol.
20. After incubating cells in fix/perm buffer for 60 min, rinse with 1× perm buffer, and spin for 5 min at 700 r.c.f. at 4 °C.
21. Resuspend in 200 μL 1× perm buffer and spin for 5 min at 700 r.c.f. at 4°C.
22. Resuspend in 50 μL 2× Fc Block in perm buffer, and incubate for 10 min at 4 °C.
23. Prepare to stain cells with intracellular antibodies of choice. Useful markers include anti-TCRβ (BD), anti-Ki67(Thermo), anti-TNF (eBioscience), anti-IFNγ (Tonbo), and anti-FOXP3 (eBioscience).
24. Add 50 μL of 2× master mix with intracellular antibodies in 1× perm buffer and incubate at 4 °C for 60 min.
25. Add 100 μL perm buffer, spin for 5 min at 700 r.c.f., and decant supernatant.
26. Resuspend with 200 μL perm buffer, spin for 5 min at 700 r.c.f., and decant supernatant.
27. Resuspend in 200 μL FACS buffer, spin for 5 min at 700 r.c.f., and decant supernatant.
28. Resuspend with 200 μL FACS buffer, and filter through Nytex.
29. Analyze samples on a BD LSRFortessa cell analyzer and use FlowJo for data analyses.

4 Notes

1. Authenticity and sterility of cell lines should be maintained by using microscopy to confirm cell morphology and animal cell lines should be frequently tested for mycoplasma contamination.
2. For the purposes of a streamlined protocol, we will use the A20 cell line as an example. However, we have also shown delayed tumor growth an immunologically colder murine colorectal cell line, CT26 (ATCC, CRL-268), and will include additional notes throughout the protocol where applicable.
3. All animal experiments were approved by the Institutional Animal Care and Use Committee (Columbia University, protocols ACAAAN8002 and AC-AAAZ4470). All procedures described here should be approved by the Institutional Animal Care and Use Committee (IACUC). All personnel should be trained (including but not limited to health clearance, humane

handling of laboratory animals, and barrier facilities) and follow the approved protocols at all times.

4. It is important to note the difference in mouse sex when performing animal experiments. This may influence the result and should be accordingly disclosed.
5. All mice should be randomized prior to treatment.
6. Statistical analysis and sample sizes were determined from previous studies [34, 37, 38].
7. For downstream treatment of CT26 tumors, clone a plasmid encoding a murine GM-CSF cytokine whereby a geneblock (IDT) encoding a *tac* promoter and the *E. coli* codon-optimized murine GM-CSF cytokine (UniPort P01587) is assembled to a linearized p246-axe/txe stabilized plasmid.
8. Integrations are stably maintained in the absence of antibiotic selection, but frequent monitoring of lysis behavior using sequencing, plate reader, or microscopy is suggested to confirm maintenance of expected lysis circuit and phenotype.
9. Addition of AHL at a final concentration of 10 nM is needed to visualize lysis using in vitro methods like microscopy or a plate reader time course.
10. To increase the optical density (OD_{600}) at which quorum and therefore lysis occurs, glucose (final concentration of 0.2%) can be added to the culture.
11. Integration of this lysis operon does not affect growth kinetics and the doubling time therefore remains similar to that of wild-type EcN (~30 min).
12. Electrocompetent SLIC strains can be prepared similarly with the added modification of supplementing all LB media with 0.2% glucose.
13. Place tube in ice during sonication to prevent samples from overheating.
14. This protocol should also be used to generate negative control lysate samples to be used in downstream studies. Therefore, lysate should be collected from the EcN-lux vector as well.
15. PBS can be used in lieu of RPMI since incubation with mammalian cells is not excessively long.
16. More information regarding the western blot method and corresponding reagents can be found in reference [32]. Briefly, proteins were probed for 1 h with primary anti-HA antibody (Roche) and 1 hour with a rat-HRP (GE Life Sciences) at room temperature. Protein was detected using chemiluminescent substrate.
17. This protocol can be repeated as described below with CT26 cells.

18. Seed enough wells to account for all negative assay and flow cytometry controls including unstained cells, media only, and compensation controls.
19. This should include lysate from both the EcN-lux-expressing PD-L1 plasmid and EcN-lux without a plasmid.
20. The PD-L1 nanobody competes for the same epitope as the 10F.9G2 antibody. More details can be found in reference [32].
21. Be sure to include single-fluorophore-only compensation controls.
22. Any mouse background can be used, but for our experiments, a 6- to 8-week-old female C57BL/6 mouse was used.
23. This volume does not need to be exact. The purpose here is to just wet the filter.
24. After extracellular staining to detect extracellularly bound nanobody, cells can be washed, fixed, and stained for CD3 and intracellular CTLA-4 to assist with analysis and controls.
25. For statistical purposes, there should be ~5 mice per group and all groups should have comparable sample sizes.
26. Each mouse will receive a 100 μl injection per flank (200 μl per mouse if grafting both hind flanks). Be mindful to have ~30% more volume than is needed for the injections because some volume will be lost in the needle.
27. If treating CT26 tumors, add SLIC-GMCSF therapeutic as well and administer an equal parts cocktail of SLIC:PD-L1nb,-SLIC:CTLA-4nb, and SLIC-GMCSF.
28. Resuspend the pellet in a 1-mL pipette to ensure adequate dislodging of the bacterial pellet and then pour 49 mL of DSPBS and invert conical multiple times to homogenize.
29. While the needle is still inside the tumor, move around the needle in small circles before very slowly pulling the needle out. Rapid removal of the needle will result in bacteria therapy squirting out of the dense tumor.
30. Due to the integration of the lysis circuit, we have previously shown that only a single intratumoral injection of SLIC therapeutic strains is needed to achieve tumor reduction in the A20 model.
31. After spotting the dilutions, let the agar plate dry uncovered near an open flame until the spots dry. Turning the plate over too quickly before letting it dry and putting it in the incubator will smear the spots and make it more difficult to identify which colonies belong to each dilution.
32. Day 6–8 is chosen as the time point range because this is when an adaptive immune response is expected to peak.

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